"Studies of the secondary metabolite content of Tasmanian *Caulerpa* species"

Ph D thesis

by

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Chemistry

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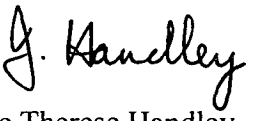
Volume 1

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of the candidate's knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

Signed: 

Jacqueline Therese Handley

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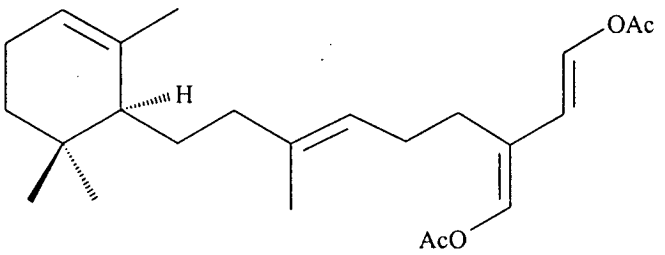
Jacqueline Therese Handley

Abstract

Five species of Tasmanian green algae have been investigated for their biological activity and secondary metabolite content. A total of thirteen novel secondary metabolites and eleven known secondary metabolites containing acetoxybutadiene, dialdehyde or related moieties have been isolated and identified.

From *Caulerpa trifaria* the novel secondary metabolites **(1)–(4)** have been characterised whilst the known secondary metabolites **(5)** – **(8)** have also been isolated.



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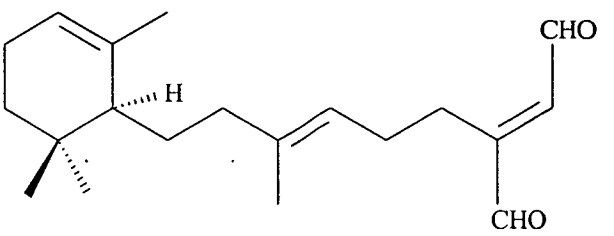

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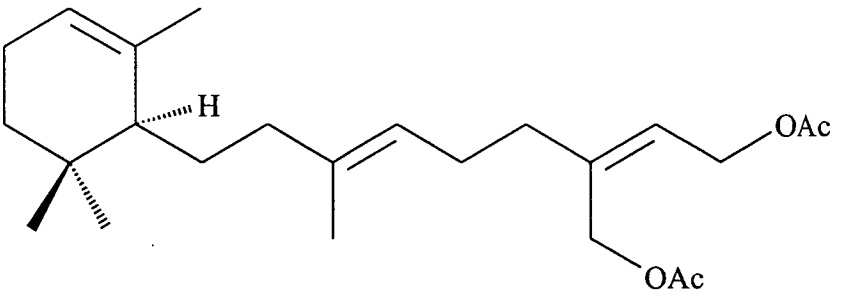

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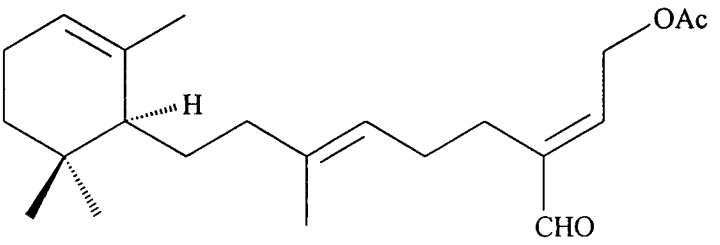

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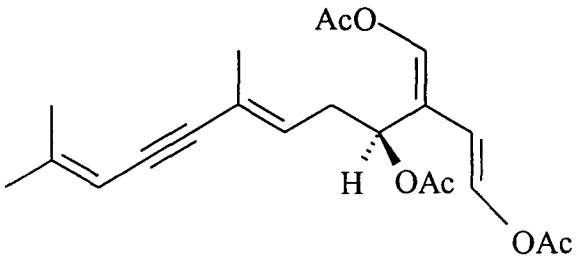

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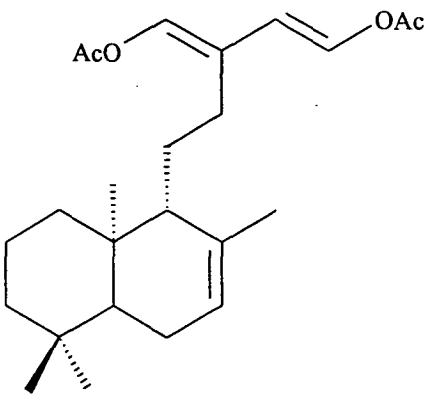

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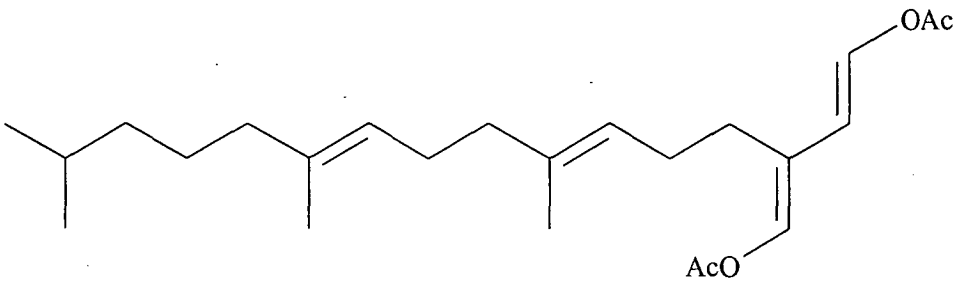

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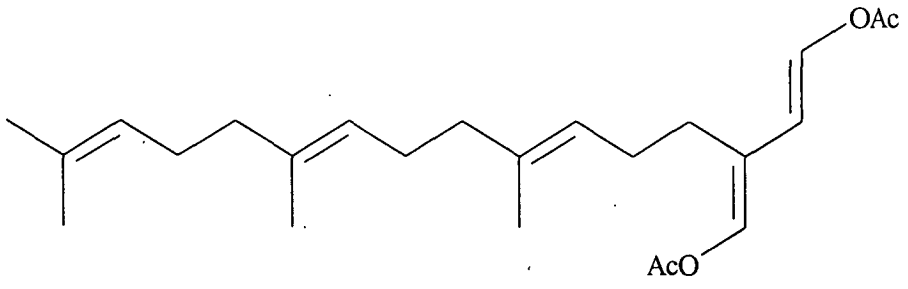
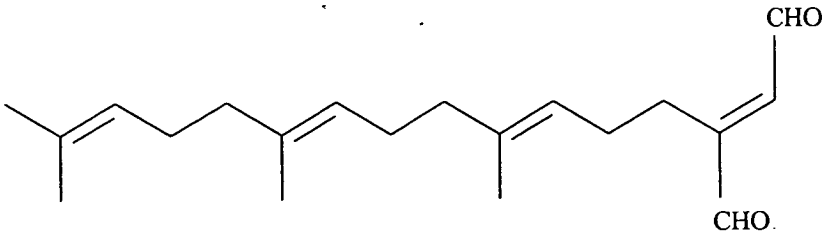


(8)

From unbranched specimens of *Caulerpa brownii* the novel secondary metabolites **(1)**, **(2)**, **(10)** – **(14)** and the known secondary metabolites **(7)**, **(8)**, **(15)** and **(16)** have been isolated. The metabolite **(9)** has been isolated for the first time as a natural product.



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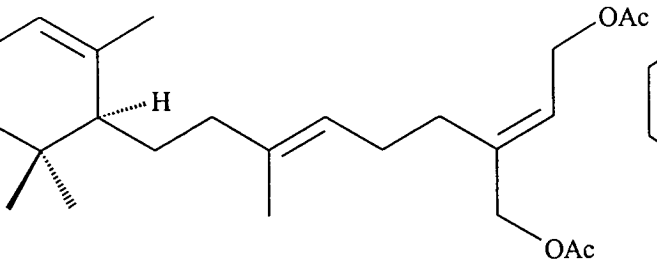

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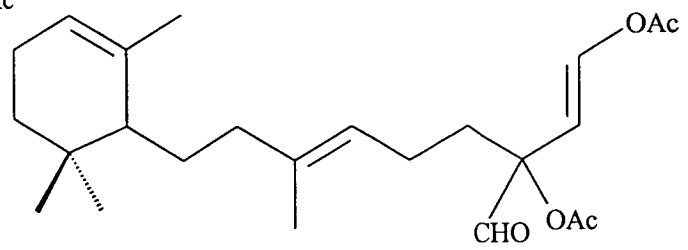

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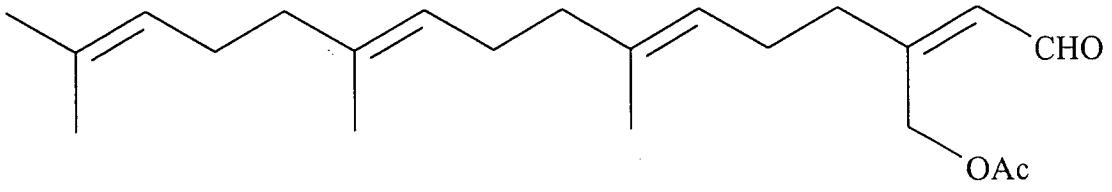

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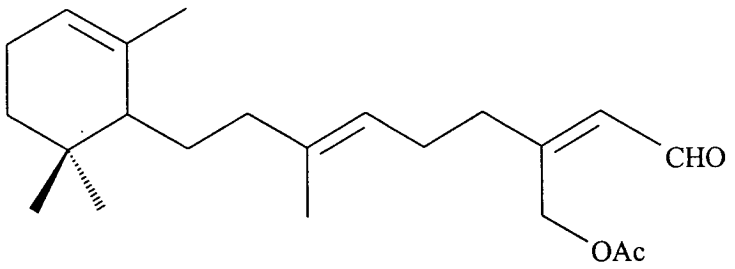

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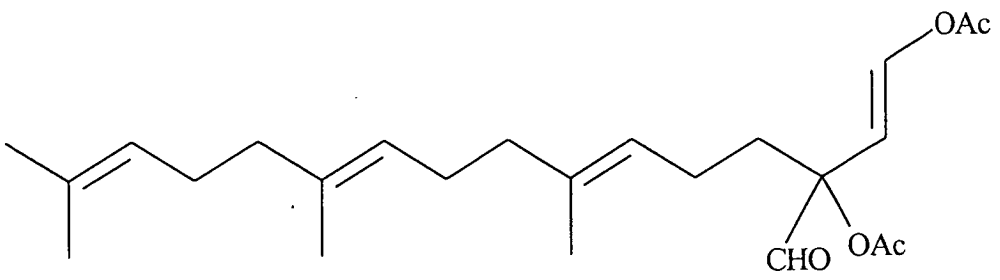

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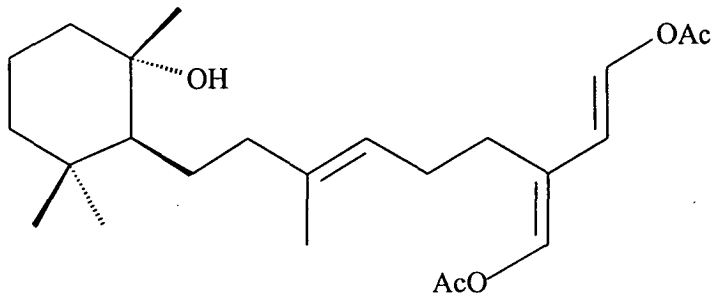

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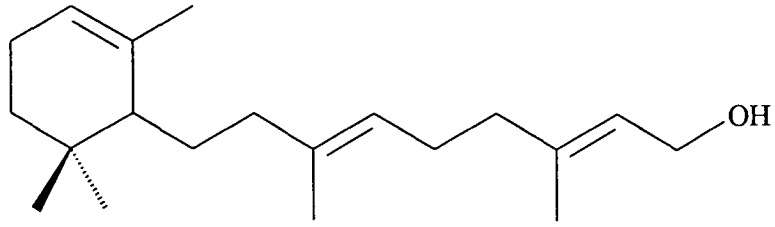
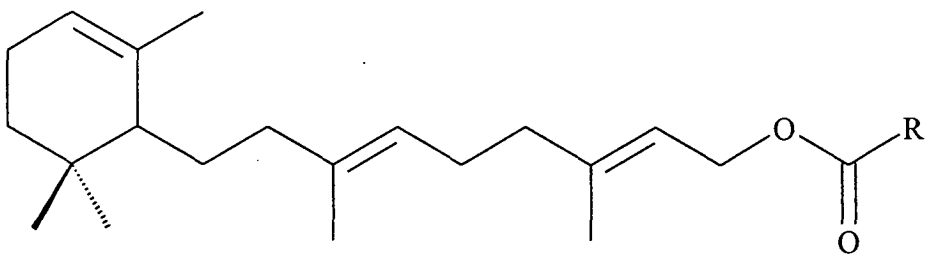


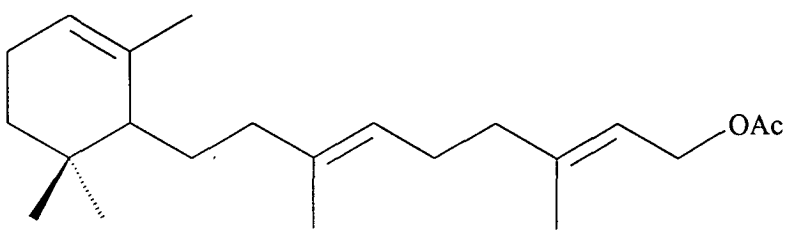
(16)

The novel terpenoid esters **(17)** and the known secondary metabolites **(16)** and **(18)** have been identified from branched specimens of *Caulerpa brownii* whilst the novel secondary metabolites **(19)** and **(20)** and the known secondary metabolites **(16)** and **(21)** have been isolated from *Caulerpa flexilis*. Methods for isolating a further six metabolites from *C. flexilis* are also described.



$R_1 = C_{19}H_{31}$-
$R_2 = C_{19}H_{29}$-
$R_3 = C_{17}H_{33}$-
$R_4 = C_{17}H_{31}$-
$R_5 = C_{17}H_{29}$-
$R_6 = C_{15}H_{25}$-

(17)



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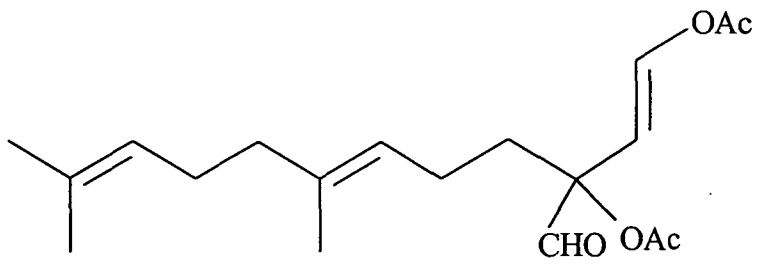

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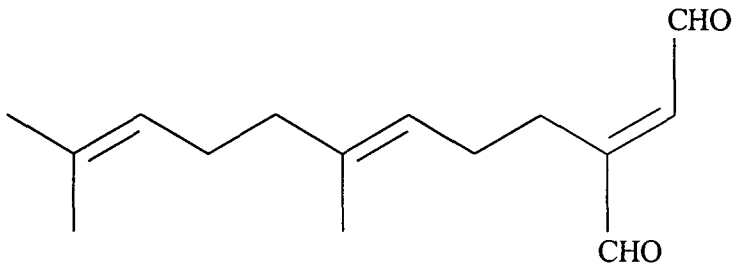

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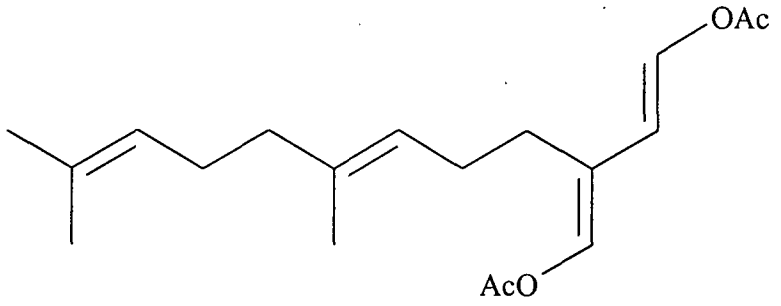
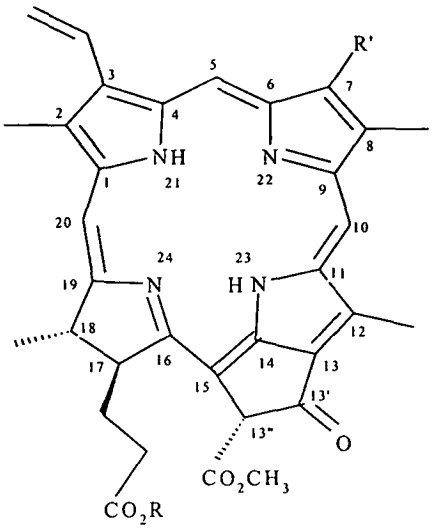


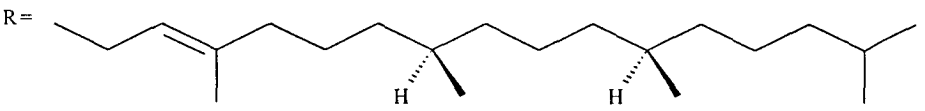
(21)

A preliminary study of *Caulerpa scalpelliformis* and *Caulerpa longifolia* has resulted in the isolation of the ecologically important secondary metabolite caulerpenyne **(5)** and associated compounds.

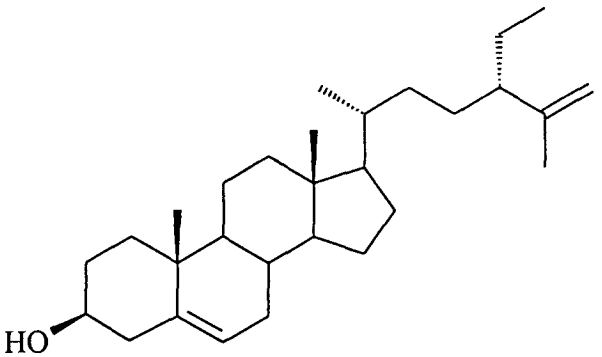
Geographic, seasonal and other factors affecting the production of secondary metabolite production in the five *Caulerpa* species are discussed whilst the biological activities of extracts of the *Caulerpa* species and selected metabolites are also described. The biological activities of the 1,4-dialdehyde, acetoxybutadiene and related moieties and the relationship between the secondary metabolites contained in the *Caulerpa* species *C. flexilis, C. trifaria* and *C. brownii* are also discussed.

The associated pigments pheophytin a and b **(22)**-**(23)** and the sterol cholesterol **(24)** have also been isolated from several of the *Caulerpa* species studied for this thesis.





(22) R' = CH_3, **(23)** R' = CHO



(24)

Acknowledgements

Difficulties are encountered during the course of any Ph D project, some of which are University based but others which in my case have impacted on my studies more severely. These latter difficulties include being diagnosed with breast cancer in April 2001 and requiring a year of surgery, chemotherapy and radiation treatment both in Tasmania and on the mainland. Ironically, my chemotherapy treatment regime included a natural product "taxotere" (obtained from the Pacific Yew tree) which has been shown in clinical trials, to improve survival rates over more conventional drugs. My cancer diagnosis curtailed the experimental phase of my Ph D project leaving an additional six compounds from *C. flexilis* close to completion and also limiting the scope of investigations into *C. scalpelliformis* and *C. longifolia.* It is only due to the support of a number of people that I am finally able to submit my thesis in March 2003.

I wish to acknowledge firstly my supervisor Dr. Adrian Blackman who has encouraged me at every turn with advice and support both on professional and personal matters. I also wish to thank Dr. Blackman's family for welcoming us into their home on many occasions and their kind thoughts during my illness. Secondly I wish to acknowledge the other members of the Marine Natural Products Group at the University of Tasmania; Jongkolnee Jongaramruong, Christian Narkowicz and Martin Hitchman for assistance in collection of seaweeds and moral support over the last six years. I would also like to thank Martin for his photographs of *Caulerpa* species and for accessing experimental data for me whilst I was writing up as a remote student in Victoria. Thirdly I wish to acknowledge the camaraderie and friendship of the staff and postgraduate students at the School of Chemistry at the University of Tasmania; in particular Anne

Kelly, Peter Traill, Di Craw, Grant Shepherdson, Jim Patel, Michaela Wegman and many others who made it an enjoyable place to be.

Fourthly I wish to acknowledge the assistance of the staff of the Central Science Laboratory at the University of Tasmania; in particular Dr. Noel Davies, Dr. Evan Peacock and Mr. Marshall Hughes for their ongoing assistance and advice with the running of g.c.-m.s., l.c.-m.s. experiments and 2 D n.m.r. experiments. I wish to also acknowledge Dr. Graham Edgar of the Zoology Department of the University of Tasmania for permission to use photographs from his book "Australian Marine Life".

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I also wish to acknowledge the unquestioning love and support that my parents Jack and Joan Handley and my siblings (Jo, Patrick, Michael and Francine) and their families have given me over what have been the most difficult years of my life to date. Finally I wish to thank my darling daughter Annie for her constant love and attention and making me realise what the important things in life really are. It has taken a long time to get to this point however I have often reminded myself that:

"It is good to have an end to journey to, but it is the journey that matters in the end."

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List of Abbreviations

Abbreviation	Full Text
DCM	dichloromethane
u.v.	ultraviolet
g.c.	gas chromatogram
m.s.	mass spectrum
p.t.l.c.	preparative thin layer chromatogram
t.l.c.	thin layer chromatogram
EtOH	ethanol
DEPT	distortionless enhancement through polarisation transfer
HMBC	heteronuclear multiple bond correlation
COSY	correlation spectroscopy
INADEQUATE	correlation through multiple-quantum coherence
HETCOR	heteronuclear correlation
n.m.r.	nuclear magnetic resonance
e.i.	electron impact
MeOH	methanol
EtOAc	ethyl acetate
Pet. ether	petroleum ether (40-60)

Chapter

One

Introduction

Contents

1.1 Marine natural products research

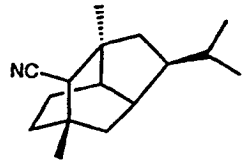
While the natural products chemistry of terrestrial organisms has played an integral part in the development of organic chemistry, study of the natural products of marine organisms has only begun in the last thirty-five years.[1] This was due to the difficulty of obtaining specimens and also to accurately identifying them. The invention of SCUBA diving however, made collection of samples possible and an increased understanding of marine biology and taxonomy has greatly aided the identification process. This has resulted in a rapid expansion of our knowledge of marine natural products since then. With over 500 000 marine species[1] there is still plenty of scope for research in this area despite some easily collected phyla having been well studied, reducing the number of new bioactive compounds being isolated from such sources.[2]

Plants produce both primary and secondary metabolites. Primary metabolites are produced by photosynthesis and are compounds which are basically involved in the life processes of all plants. Secondary metabolites are non-essential to these life processes but are needed for species survival and are characteristic of a particular biological group.

The synthesis of these secondary metabolites is related to the evolution of the species. Plants which are easily recognisable (apparent) use two different strategies to defend themselves; either they make themselves tough and unpalatable which is costly in metabolic terms or they synthesise highly active repellants.[3] Unapparent plants (less recognisable) use a less expensive synthetic strategy and produce a small quantity of active toxin that interferes with the metabolism of the herbivores.[4]

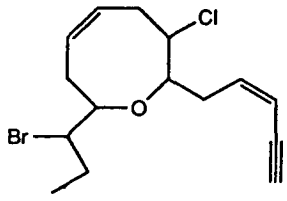
It is now well established that organisms may sequester and store toxic compounds from the plants they feed on and use them for defence. Occasionally the

compounds are modified by the organism.[5,6,7] For example, the brightly coloured nudibranch *Phyllidia varicosa* lacks physical protection however it is rarely eaten by fish. It secretes a volatile, strongly smelling, ichthyotoxic sesquiterpenoid **(1)** which protects it from predation. *P. varicosa* feeds on a sponge *Hymeniacidon* sp. with the same smell, which produces **(1)** in substantial amounts.[8]



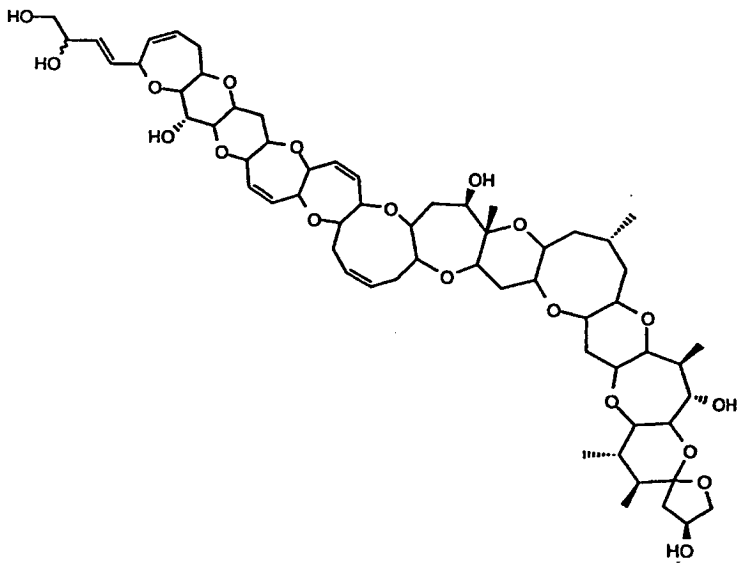
(1)

The organohalo compound **(2)** is found in the red alga *Laurencia pinnatifida*.[9] An isomer of **(2)** is found in the sea hares *Aplysia* spp. whose diet includes *L. pinnatifida*.[10]



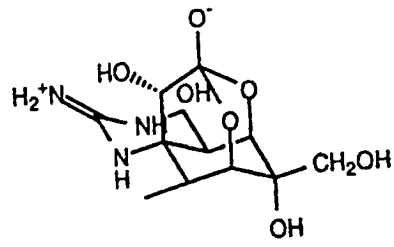
(2)

The study of marine natural products has resulted in the isolation of secondary metabolites with diverse and novel chemical structures possessing potent biological activities.[11] The structure of ciguatoxin **(3)** involved in seafood poisoning took thirty years and high field NMR spectroscopy before being solved.[12] Ciguatoxin is a dinoflagellate metabolite which passes up the food chain into the fish and is present in very low concentrations (180 kg of fish yielded 100 μg of **(3)**).



(3)

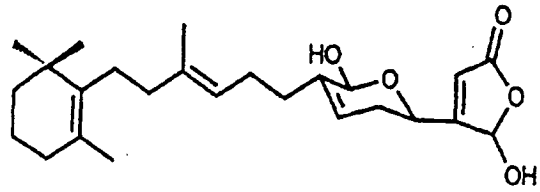
Tetradotoxin (4) is found in the puffer fish (fugu), which is prized as a delicacy in Japan and has also been isolated from a red alga, the blue-ringed octopus, crabs and salamanders.[13] Tetradotoxin has since been found to be a bacterial metabolite[14] and acts as a sodium blocker in nerve cells causing paralysis. Clinically it has been used to relax muscle spasms and also in studies on nerve cells.[15]



(4)

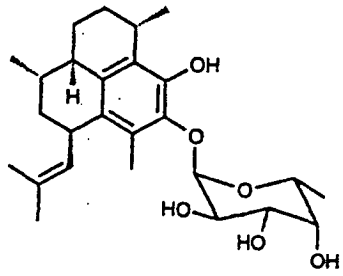
In the last fifteen years marine natural products research has focused on the isolation of compounds with pharmaceutical potential, particularly anticancer agents.[2,16,17] A number of marine natural products have subsequently been approved for clinical and pre-clinical studies.[16]

Arabinose-nucleosides from the Caribbean sponge *Cryptotethya crypta* have led researchers to synthesise analogues; ara-A (Vidarabin, Vidarabin Thilo^{R}) and ara-C (Cytarabin, Alexan^{R}) with improved antiviral and anticancer activity.[11] The sesquiterpenoid manoalide **(5)** obtained from the sponge *Luffariella variabilis* (Thorectidae) is a potent inhibitor of phospolipase A_2 (PLA_2) and also functions as a calcium channel blocker. A number of derivatives of **(5)** have been synthesised and are undergoing clinical trials.[11]



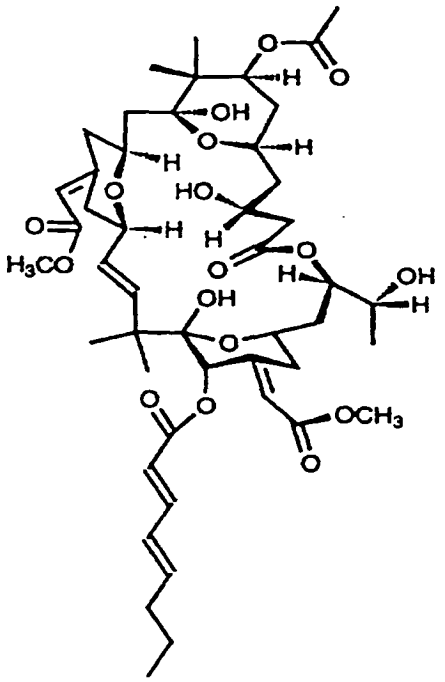
(5)

Pseudopterosins from the Caribbean gorgonian *Pseudopterogorgia elisabethae* (Ggorgoniidae) have shown promise.[11] Pseudopterosin E **(6)** combines low toxicity and potent anti-inflammatory activity and is currently in phase 1 clinical trials.[18]



(6)

Bryostatins from the bryozoan *Bugula neritina* (Bugulidae) were first discovered twenty five years ago. Bryostatin 1 **(7)** has completed phase 1 clinical trials in the USA and is in phase 2 trials in Europe as an anticancer agent.[18]



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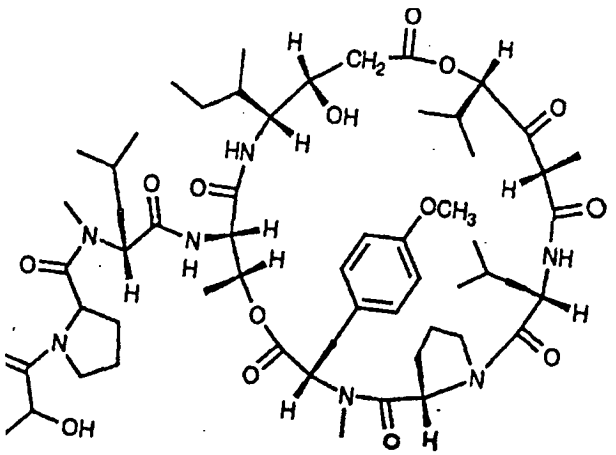
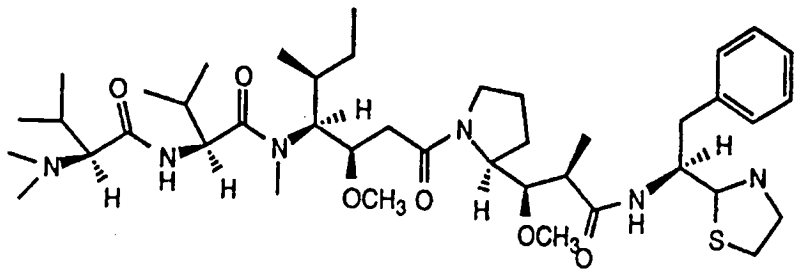
Didemnin B **(8)** from the tunicate *Tridendum solidum* (Didemnidae) has antiviral, immunosuppressive and potent cytotoxic properties and is in phase 1 and 2 clinical trials as an anticancer agent.[18]



(8)

Dolastatin 10 (**9**), a peptide from the sea hare *Dolabella auricularia* (Alysiidae), ecteinascidins such as ecteinascidin 743 (**10**) from the tunicate *Ecteinascidia turbinata* (Perophoridae), haloman (**11**) from the red alga *Portieria hornemanii* (Rhizophyllidacae) and halichondrin (**12**) isolated from several sponges are also undergoing preclinical evaluation.[20, 21]



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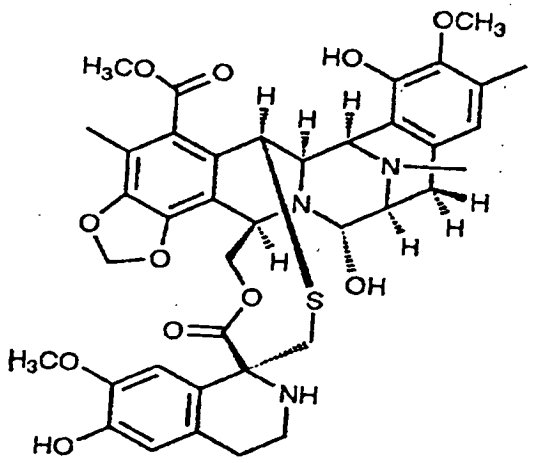

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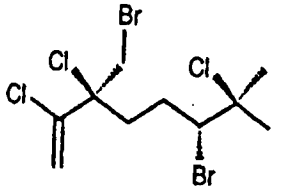

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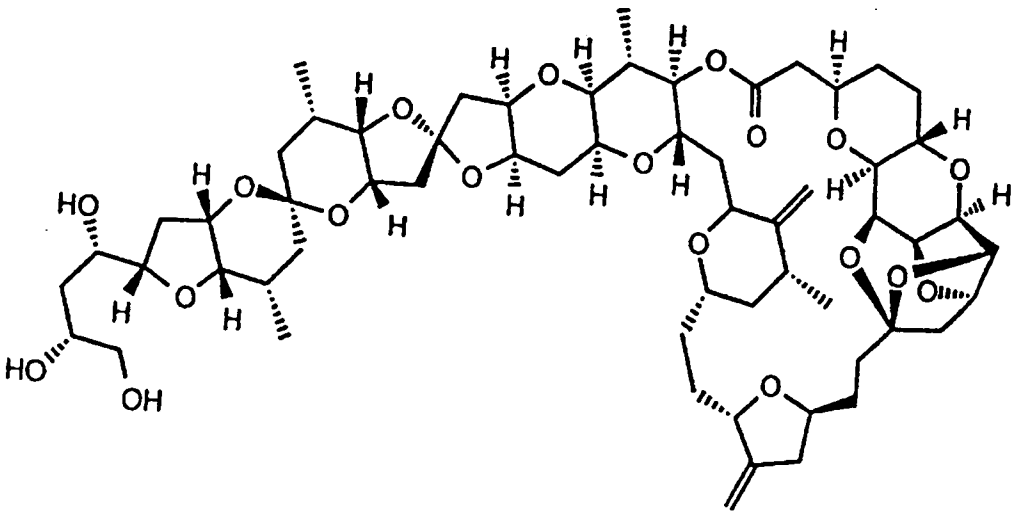


(12)

Marine organisms generally produce complex organic extracts often containing trace amounts of each component. Thus the isolation of pure metabolites can be difficult, involving large collections and elaborate purification sequences. Also seemingly identical colonies of organisms such as sponges or algae may sometimes have different chemical compositions. Despite such difficulties the unique structures and physiological activities of many marine metabolites continues to fuel investigations into marine natural products.[2]

Screening crude extracts using bioassays followed by bioactivity-directed separations is the most successful method of isolating compounds with interesting bioactivity. This requires access to benchtop assays such as the brine shrimp assay or the lettuce seedling assay used in this thesis or sending extracts away to an outside agency such as Norvatis or laboratories that employ NCI assays or specific assays such as topoisomerase inhibition or protein kinase C binding.[17]

It can take many years for a compound with promising activity to undergo testing and many fail due to poor transfer of activity to live models or unwanted side effects. Limited supply can also pose a problem if a natural product is used for extensive testing, since large collections can be environmentally damaging.[19] In these cases synthesis (as with dolastatin 10[20]) or culturing the organism (as with the bryostatins[21]) can be the only way of generating supply.

The marine natural products chemist therefore needs to collect sufficient material and to isolate what may be a very small amount of compound from a complex mixture in a multistep procedure. Access to suitable assays are useful at different points in this process. Once isolated the pure metabolite needs to be structurally identified, which despite current NMR spectroscopic techniques can be difficult, particularly when small quantities are involved or the metabolite is unstable.

Historically the majority of secondary metabolites have been isolated from macroalgae, coelenterates, echinoderms and sponges, chiefly due to the abundance and ease of collection of these organisms. Recently the contribution from macroalgae has decreased whilst sponges have become the dominant source of new compounds. Sponges have a wider range of biosynthetic capabilities than any other group of marine invertebrates thus giving rise to a diversity of structural types. The modest contributions by ascidians and microbes is also increasing due to the potent metabolites produced by the former and the potential of culturable media as renewable sources of pharmaceuticals for the latter. Comprehensive investigations of the saponins produced by starfish have also increased the number of metabolites produced by echinoderms.[24] The number of metabolites isolated from different marine organisms partially depends on the availability

of the marine organism and also the specific interests of investigators. With so many marine species still to be investigated it is likely that marine organisms will continue to be significant sources of natural products for decades to come.

1.2 Previous studies of green algal secondary metabolites

Marine algae are photosynthetic plants relatively simple in structure and adapted for a marine environment. They fall into two major categories: the large macroalgae occupying the littoral zone and benthic zones and the microalgae occupying both the littoral and benthic zones and also throughout the photic region of ocean waters as phytoplankton. Figure 1.1 shows a basic scheme outlining where the algae fit into the classification of living organisms.

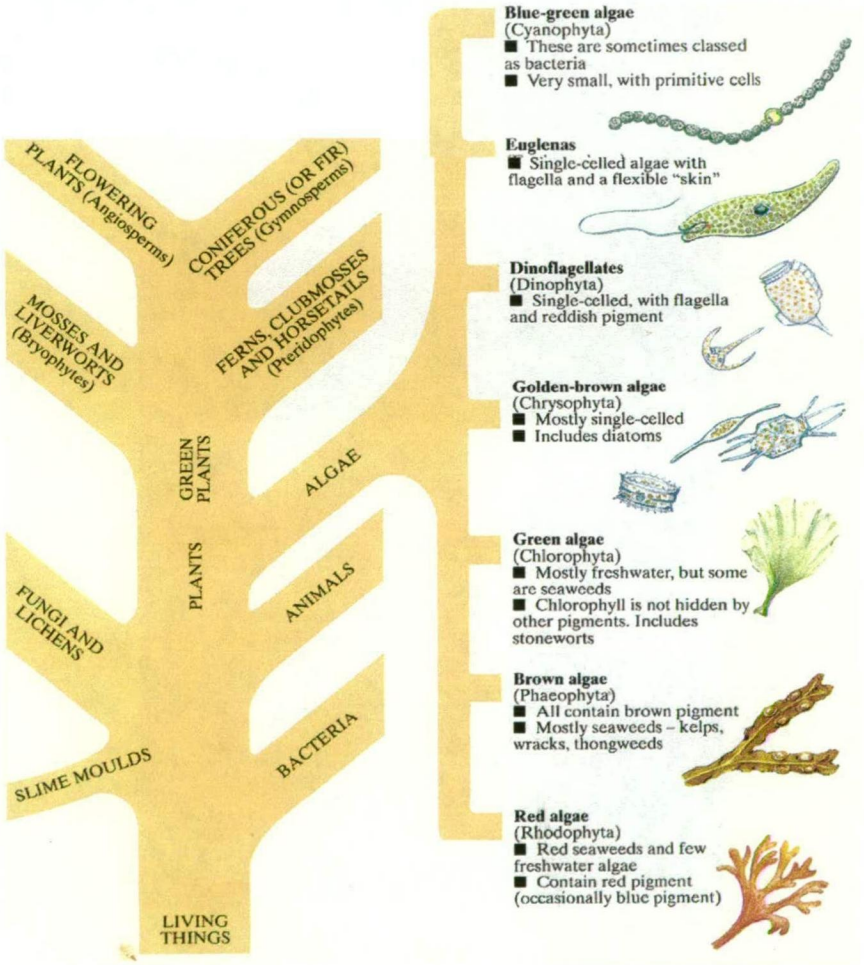


Figure 1.1 Basic Classification Scheme[25]

The further classification of algae is based on the pigments used in gathering light energy for photosynthesis; for example all plants contain chlorophyll *a* and β–carotene but the accessory pigments vary. The other important characteristics used for classification are the nature of the storage products and the number, type and arrangement of the flagella in motile cells.[23] Table 1.1 shows the classification of algae based on pigments.

Division	Popular name	Major accessory pigments	Storage products	No., arrangement, type of flagella
Chlorophyta	Green algae	Chlorophyll *b*	Starch (amylose + amylopectin)	2, 4 or many equal, anterior, smooth
Charophyta	Charophytes	(as Chlorophyta, but distinguished by vegetative and reproductive morphology)		
Euglenophyta	Euglenoids	Chlorophyll *b*	Paramylon	1 anterior, tinsel
Phaeophyta	Brown algae	Chlorophyll c_1 + c_2, fucoxanthin	Laminaran	2 unequal, lateral, smooth + tinsel
Chrysophyta	Yellow-brown or golden-brown algae	Chlorophyll c_1 + c_2, fucoxanthin	Chrysolaminaran	1 – 3 anterior, various
Pyrrhophyta	Dinoflagellates	Chlorophyll c_2, peridinin	Starch	2 equal, smooth
Cryptophyta	Cryptomonads	Chlorophyll c_2, phycobilins	Starch	2 equal, lateral, both tinsel
Rhodophyta	Red algae	Phycoerythrin ± phycocyanin	Floridean starch (amylopectin)	None
Cyanophyta	Blue-green algae	Phycocyanin ± phycoerythrin	Myxophycean starch (glycogen-like)	None

Table 1.1 Primary classification of algae[2]

Plants can also be classified on a morphological basis or on an ecological basis according to their habitat; for example marine plants require regular or continous immersion in seawater.[25] This thesis is primarily concerned with marine green algae (Chlorophyta); details of Chlorophyta are shown in Table 1.2.

ORDER	family	GENUS
TETRASPORALES	Palmellaceae	*PALMOCLATHRUS*
ULOTRICHALES	Ulotrichaceae	*ULOTHRIX* *URONEMA*
CHAETOPHORALES	Chaetophoraceae Chroolepidaceae	*ENTOCLADIA* *PILINIA*
ULVALES	Ulvaceae	*PERCURSARIA* *ULVARIA* *ULVA* *BLIDINGIA* *ENTEROMORPHA*
PRASIOLALES	Prasiolaceae	*PRASIOLA* *ROSENVINGIELLA*
CLADOPHORALES	Cladophoraceae	*RHIZOCLONIUM* *CHAETOMORPHA* *APJOHNIA* *CLADOPHOROPSIS* *CLADOPHORA* *WITTROCKIELLA*
	Anadyomenaceae	*MICRODICTYON* *ANADYOMENE* *STRUVEA*
ACROSIPHONIALES	Acrosiphoniaceae	*UROSPORA*
SIPHONOCLADALES	Valoniaceae	*DICTYOSPHAERIA*
CODIALES	Codiaceae	*CODIUM*
CAULERPALES	Udoteaceae	*PSEUDOCHLORODESMIS* *CHLORODESMIS* *HALIMEDA* *PSEUDOCODIUM* *CALLIPSYGMA* *RHIPILIA* *RHIPILIOPSIS* *AVRAINVILLEA*
	Caulerpaceae	*CAULERPA*
DERBESIALES	Bryopsidaceae Derbesiaceae	*BRYOPSIS* *DERBESIA [& HALICYSTIS]* *PEDOBESIA*
DASYCLADALES	Dasycladaceae	*DASYCLADUS*
	Polyphysaceae	*ACETABULARIA* *POLYPHYSA*

Table 1.2 Orders, families and genera of marine green algae.[84]

Relatively few chemical studies have been carried out on marine green algae compared to red and brown algae.[26] This is due in part to the fact that many of the secondary metabolites are very unstable and difficult to isolate. It has been found necessary to extract the fresh algae and immediately purify the extracts in order to isolate the secondary metabolites and determine their chemical and biological activities. Most of

the research on this group of marine algae has been carried out over the last twenty years. The following discussion reviews the literature on green algal metabolites up until February 2001.

Most green algae are tropical with members of the order Caulerpales being widely distributed on coral reefs and seagrass beds throughout the tropical oceans.[27] The order Caulerpales is subdivided into the families Caulerpaceae, containing the large genus *Caulerpa,* and the Udoteaceae containing amongst others the genera *Chlorodesmis, Halimeda, Avrainvillea, Penicillus, Tydemania* and *Udotea*[27] (Table 1.2). It is this order of green alga that has been most thoroughly examined. The families Dasycladaceae and Claddophoraceae have also been investigated chemically with several non-terpenoid secondary metabolites being isolated.

In areas of high algal growth chemical studies of green algae have been unproductive. However in the tropics and some temperate areas where feeding pressure is intense, many species of green algae have been shown to be low preference items in the diet of herbivores such as fishes and sea urchins.[28] Whilst calcification provides an effective physical defense against herbivores investigations have shown that the unique secondary metabolites produced by these algae also play a role in their chemical defense.[28]

Unprecedented secondary metabolites have been found in all the algal species studied from the order Caulerpales. The majority of new compounds that have been discovered from these families are sesquiterpenoids and diterpenoids often possessing enol acetate and aldehyde functional groups. Many of these metabolites possess a terminal bis-enol acetate moiety found uniquely in this group of marine algae. This moiety is an acetylated

bis-enol form of the 1,4-dialdehyde moiety which possesses high biological and chemical reactivity.

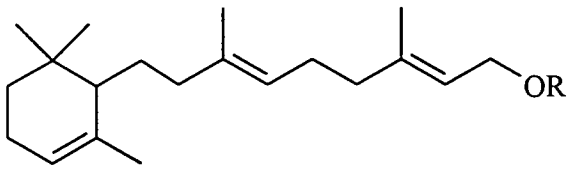
The most toxic and deterrent compounds that have been isolated from terrestrial plants and insects have been found to contain aldehyde groups.[29] Aldehyde groups, particularly unsaturated aldehydes are powerful electrophiles which can react with proteins in a number of ways to deactivate and interfere with enzmye function. Aldehydes can react with protein primary amines to form Schiff bases (imines), or nucleophiles such as sulfhydryl groups or alcohols can add to the β-carbon of unsaturated aldehydes in a "Michael Addition" reaction.[30] However not as much information is available regarding the chemical and biological reactivity of the bis-enol acetate functionality. Addition reactions to the unsaturation may be possible and *in vivo* hydrolysis could occur forming the corresponding 1,4-dialdehyde.[30]

Family Caulerpaceae

The species in the genus *Caulerpa* can be divided into two main groups. The bilateral leaf or frond-like species showing flattened ramuli in two rows are usually found in quiet waters below the littoral zone. In contrast the radially branched species possessing cylindrical closely packed ramuli are typically found in shallower and brighter parts of the littoral zone. There are exceptions to this classification, for example *C. sertularioides* is a shallow water high light plant which is bilateral but possesses cylindrical ramuli.[31]

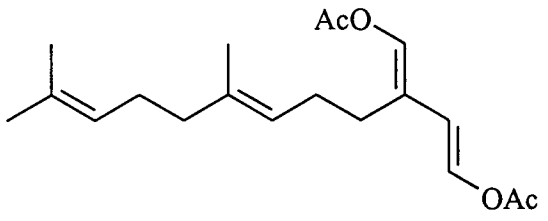
Prior to 1976 investigations into Caulerpaceae had resulted in the isolation of triterpenes[32], xanthophylls[33] and nitrogen containing compounds.[34,35] An investigation by the Blackman research group into a Tasmanian collection of *Caulerpa brownii* in 1976

identified a diterpene alcohol caulerpol **(13)** as 9.5 % dry weight of the seaweed plus the acetate **(14)**.[32]



(13) R = H **(14)** R = Ac

Studies of other Australian *Caulerpa* species by Blackman and Wells resulted in the isolation of flexilin **(15)**, a sesquiterpenoid from *C. flexilis* and trifarin **(16)**, a diterpenoid from *C. trifaria*.[36]



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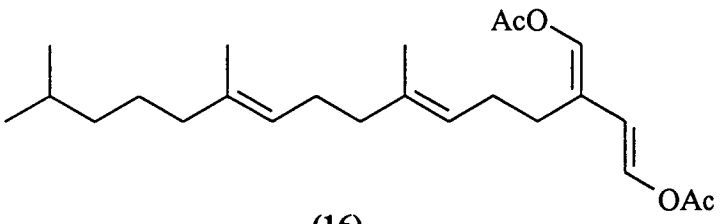
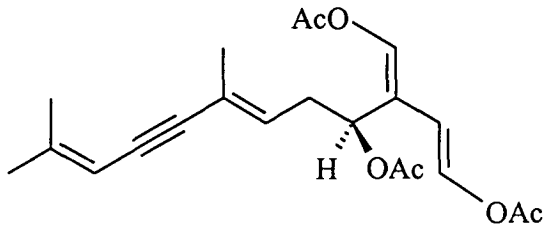


(16)

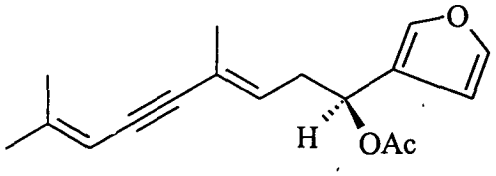
These secondary metabolites were the first natural products identified possessing the 1,4-diacetoxybuta-1,3-diene (bis-enol) moiety. This functionality has subsequently been shown to be a characteristic feature of the majority of green algal metabolites.

In 1978 investigations into *Caulerpa prolifera,*[37] widely distributed in the Mediterranean Sea identified an acyclic sesquiterpenoid caulerpenyne **(17)** as the major secondary metabolite (22% of the lipid extract). Subsequently caulerpenyne has been isolated from a number of other species of the *Caulerpa* genus common in the tropical Pacific ocean and the Caribbean Sea such as *C. racemosa, C. mexicana, C. taxifolia, C. sertularioides, C. paspaloides, C. lanuginosa, C. cupressoides* and *C. verticillata.*[37]

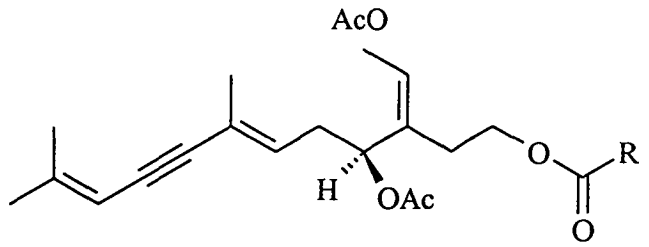


(17)

Further work in 1981 elaborated furocaulerpin **(18)** structurally related to **(17)** however containing a monosubstituted furan ring. In 1982 a didehydroderivative of caulerpenyne containing fatty acid residues **(19)** was isolated and identified.[37]



(18)



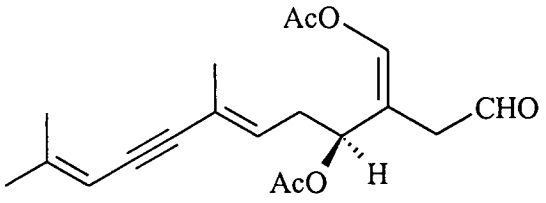
R= fatty acid residues

(19)

Caulerpenyne **(17)** has been found to be toxic and a feeding deterrent against potential grazers such as molluscs or sea urchins,[38, 39] however it does not defend the alga against tropical herbivorous fishes.[40]

The predator-prey relationship between *C. prolifera* and the three shelled sacoglassans *Oxynoe olivacea*, *Cylindrobulla fragilis* and *Lobiger serradifalci* was investigated by Gavagnin[41] in order to clarify the role of the secondary metabolites in chemical defence in both the alga and the molluscs.

A defensive role due to the presence of a protected 1,4-dialdehyde moiety had been previously suggested for caulerpenyne **(17)**. The three sacoglossans live closely associated with *C. prolifera* despite this protection. The Thyrrhenian *O. olivacea* was found to be able to modify the algal metabolite **(17)** into the more toxic oxytoxin-1 **(20)** and oxytoxin-2 **(21)**.



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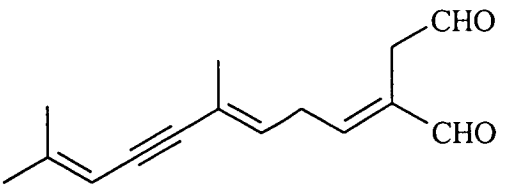
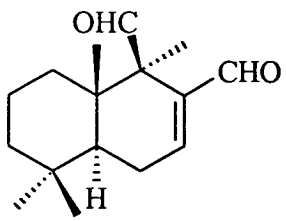


(21)

Populations of *O. olivacea* from the Spanish coast and populations of *C. fragilis* and *L. serradifalci* were found to adopt the same defensive strategies. Caulerpenyne **(17)** was transformed initially into oxytoxin-1 **(20)** in the parapodia of *L. serradifalci* and into the tail of *O. olivacea* and then in *O. olivacea* and *Cylindrobulla fragilis* to oxytoxin-2 **(21)**; containing the 1,4-dialdehyde moiety present also in polygodial **(22)** a potent antifeedant.[42]



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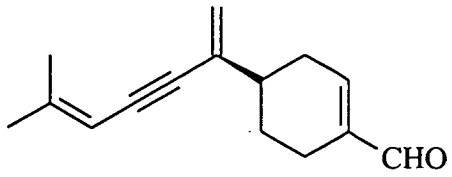
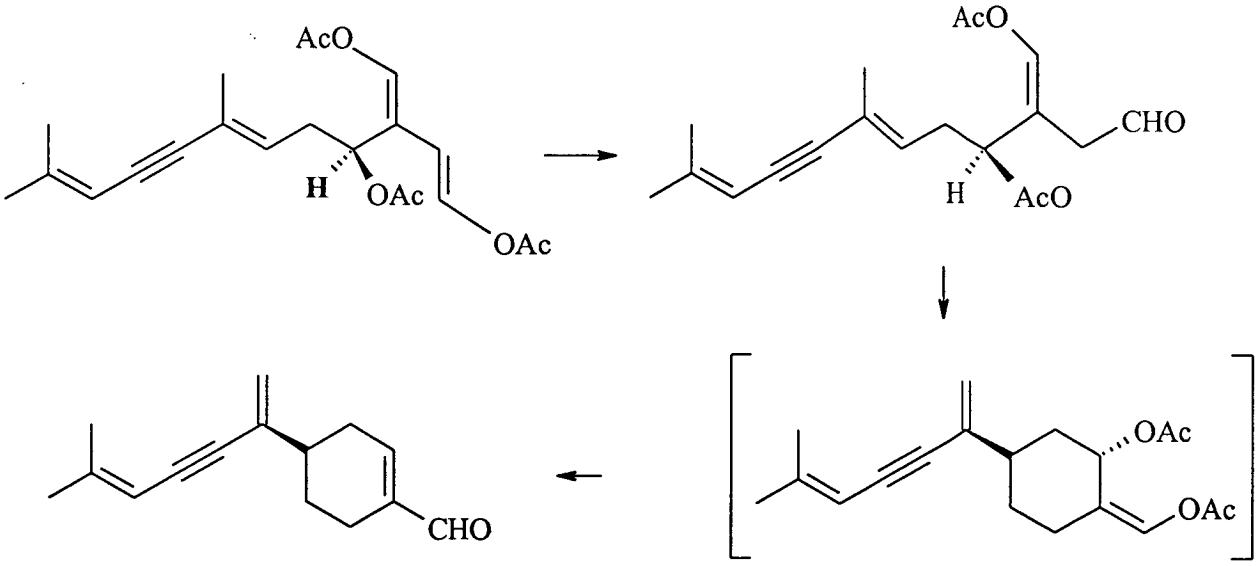


(23)

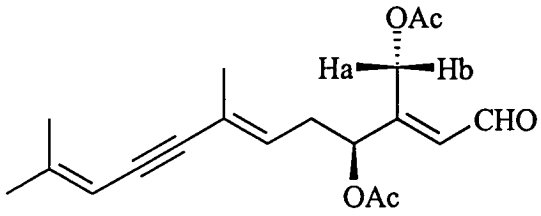
A further caulerpenyne derivative **(23)**, a highly unstable terpene, was isolated from the mucus of the Indo-Pacific mollusc *Volvatella* sp. in June 1998.[43] A biosynthetic pathway (Scheme 1.1) was proposed for the conversion of caulerpenyne into **(23)**. This mollusc feeds on *Caulerpa* species which were found to contain caulerpenyne but not the terpene **(23)**.



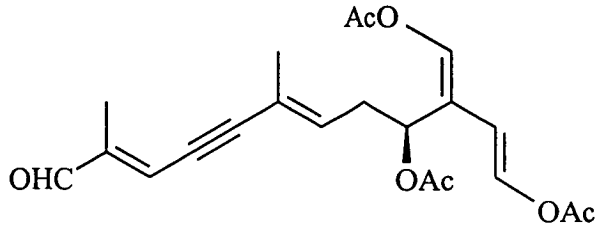
Scheme 1.1

Caulerpa taxifolia, a tropical seaweed which has recently invaded the Mediterranean contains caulerpenyne in large amounts, (greater than in the tropics) accompanied by other sesqui- and mono-terpenes.[44] This alga is resistant to winter temperatures and has an efficient system of reproduction leading to more vigorous development than in the tropics. There are concerns that *C. taxifolia* could colonise the

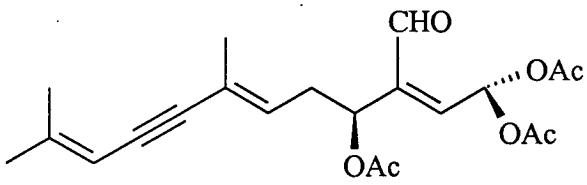
whole Mediterranean. In 1992 Guerriero and co-workers investigated collections of *C. taxifolia* from the Cap Martin and Monaco regions of the Mediterranean. This investigation isolated oxytoxin-1 **(20)** and the novel aldehyde compounds taxifolial A-D **(21)**-**(24)**.[44]



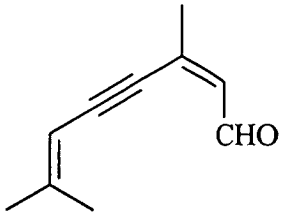
taxifolial A
(21)



taxifolial B
(22)

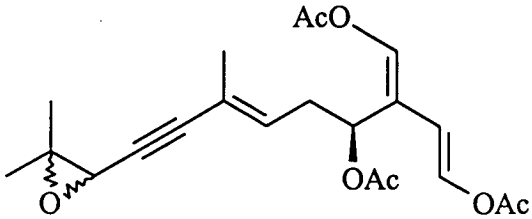


taxifolial C
(23)



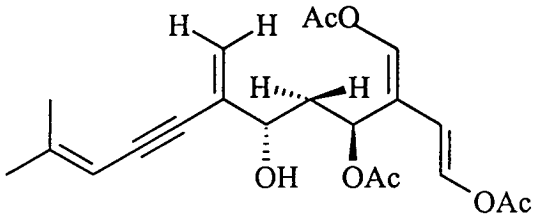
taxifolial D
(24)

The extract of *C. taxifolia* was found to inhibit the growth *in vitro* of marine bacteria and was cytotoxic towards marine ciliate protists. The most active terpene was found to be 10,11-epoxycaulerpenyne **(25)**.[45]

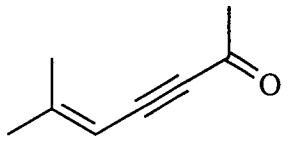


(25)

Further investigation by Guerriero and co-workers into the Cap Martin collections yielded **(26)** a strongly bioactive and biogenetically significant hydroxylated terpene and a truncated sesquiterpene taxifolione **(27)**.[45] The terpene **(26)** had the highest antibacterial activity of all terpenes isolated from *C. taxifolia*.



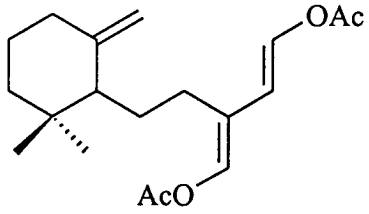
(26)



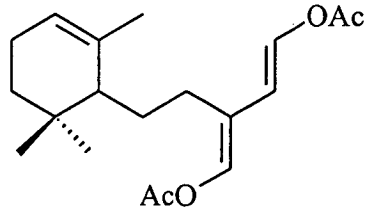
(27)

A more recent investigation[48] into the more polar fractions of *C. taxifolia* has resulted in the isolation of water-soluble stable enols and polarglycoglycerolipids. Neither of the families of compounds isolated were found to be biologically active.

A number of monocyclic sesquiterpenoids have been reported from species of *Caulerpa*. The major metabolites isolated from *C. bikinensis* (Palau) and *C. flexilis* var. *muelleri* (Western Australia) were the olefin isomers **(28)** and **(29)**.[37]

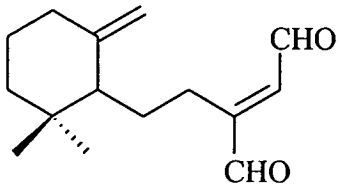


(28)

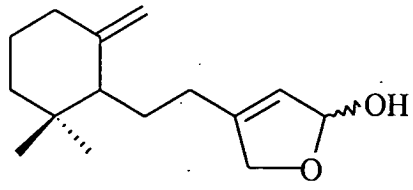


(29)

The minor metabolites isolated from *C. bikinensis* were the dialdehyde **(30)** and the butenolide **(31)**. In contrast the acetoxy-aldehyde **(32)** was the minor metabolite isolated from *C. flexilis* var. *muelleri*.[37]



(30)



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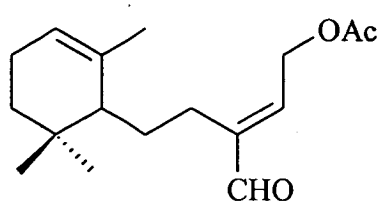
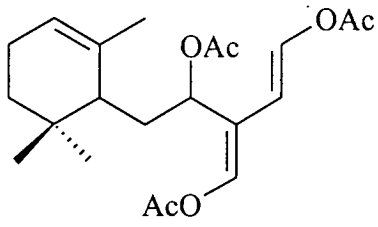


(32)

Florida collections of *C. ashmeadii* have elaborated the related monocyclic sesquiterpenoids **(33)-(36)**.[37] The presence of fatty acid esters in *C. ashmeadii* indicated that the bioactive compounds could be stored or transported as fatty acid ester-conjugates thus rendering the compounds less reactive and reducing their *in vivo* toxicities.



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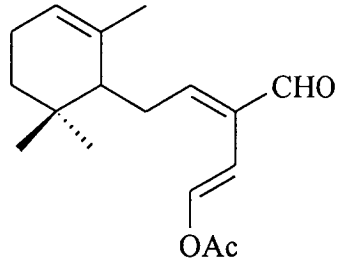

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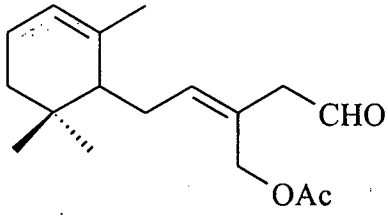

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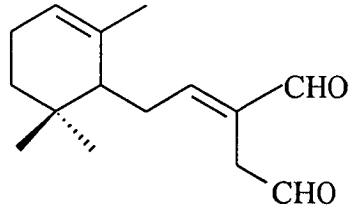
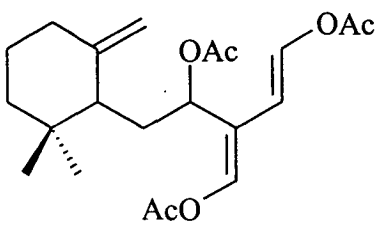


(36)

Nine monocyclic sesquiterpenoids have been isolated from the Tasmanian green alga *C. obscura*; four of these **(37)-(40)** were novel. Compounds **(37)** and **(38)** were active in the brine shrimp assay.[47]



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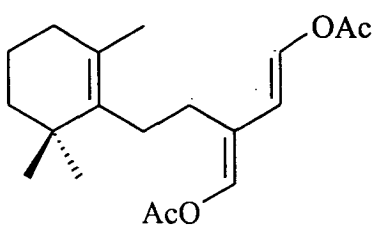

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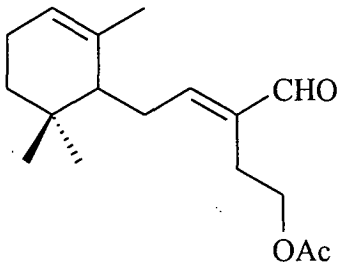

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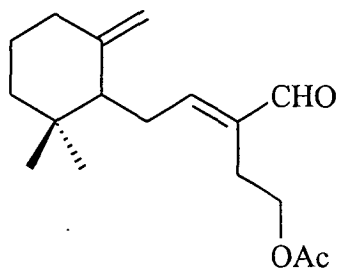
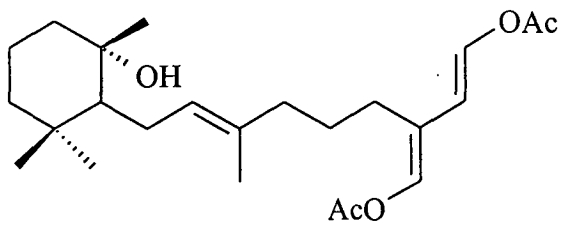


(40)

Further research into Australian *Caulerpa* species has yielded two cyclic diterpenoids possessing the terminal bis-enol acetate functionality. Metabolite **(41)** was isolated from a Victorian collection of *C. brownii* whilst the bicyclic diterpenoid **(42)** was reported from a Western Australian collection of *C.trifaria.*[48]



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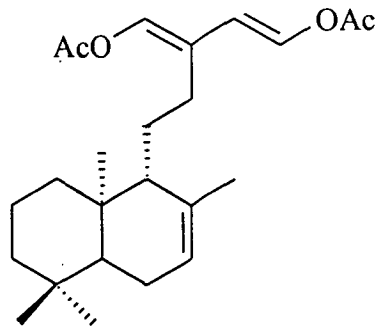
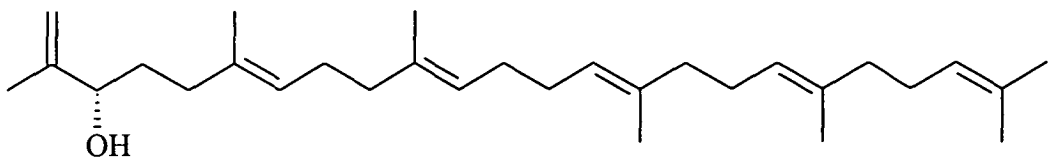


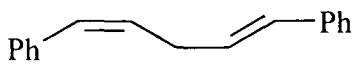
(42)

C. prolifera from the Mediterranean was also found to contain squalene derivatives including squalene, (3*S*)-squalene-2,3-epoxide, (6*S*,7*S*)-squalene-6,7-epoxide, (10*S*,11*S*)-squalene-10,11-epoxide and the alcohol **(43)**.[49]



(43)

Five unusual aromatic compounds **(44)**-**(48)** were isolated from *C. racemosa* from the Indian Ocean.[50]



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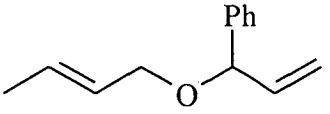

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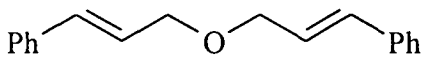

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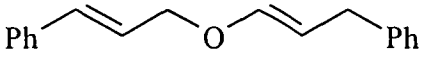

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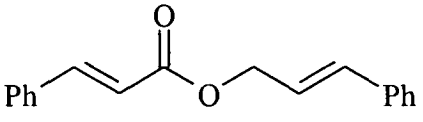
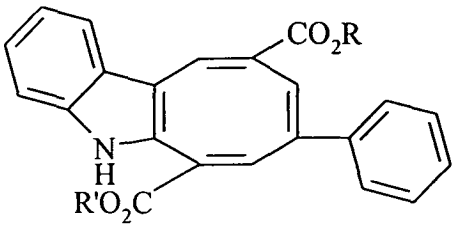


(48)

Pigments

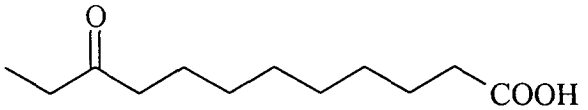
One of the first compounds isolated from the genus *Caulerpa* was the yellow-orange pigment caulerpin **(49)** from the Phillipines. *C. racemosa* and *C. sertulariodes* both yielded this compound derived from indole synthesis and it has since been found in over 50 percent of the *Caulerpa* species invesigated.[49]



(49) $R=R^1=CH_3$ **(50)** $R=R^1=H$

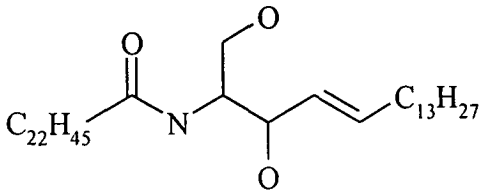
(51) $R= CH_3$, $R^1=H$

Caulerpin is generally associated with the radially branched shallow water species which posssess tightly branched whorlets[31] and has also been found to be a plant growth regulator.[51] Two analogues of caulerpin **(50)** and **(51)** were identified by Aananjaneyula and co-workers in 1991[52] from *C. racemosa* collected off the Visakhapatnam coast (India). A new keto-carboxylic acid **(52)** was also identified.



(52)

A novel ceramide **(53)** from *C. sertulariodes* has been identified[83] whilst more recently a new bis indole caulersin **(54)** has been isolated and identified from *C. serrulata* collected from the Xisha Islands in the South China sea.[53]



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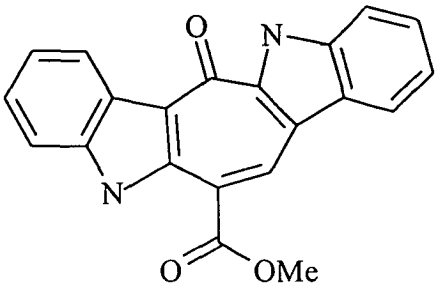
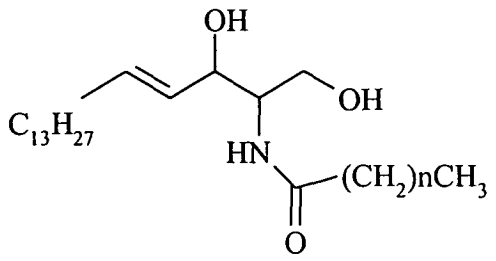


(54)

Early research into *Caulerpa* species elaborated calerpicin **(55)** as a minor metabolite from several species.[49] Caulerpicin proved to be a mixture of *N*-acylsphingosines with conflicting chemical evidence emerging about their structures.



(55)

The acute toxicity of caulerpin **(49)** and calerpicin **(55)** has been studied in mice.[54] The lack of toxicity indicates that neither compound is responsible for the toxic symptoms observed after ingestion of some Caulerpean algae. These toxic effects (numbness of tongue, cold sensation in hands and feet, difficulty in breathing and loss of balance) are similar to ciguatera poisoning. Comparative studies of the extracts of *C. scalpelliformis* however showed the aqueous extract to be poisonous to mice (100% mortality by the intraperitoneal route).[54]

C. scalpelliformis has also invaded a sponge-dominated reef in Botany Bay (New South Wales). A diving survey carried out over 1985 to 1988 did not detect this species however a 1997 investigation[55] documents its rapid expansion into deep reef habitats at the southern entrance to Botany Bay. The investigators (A. R. Davis and co-workers) also reported a significant decline in the number of sponges, colonial ascidians and bryozoans coincident with the rapid spread of *C. scalpelliformis*.

The fatty acid composition of a number of species of the genus *Caulerpa*; *C. brachypus*, *C. cupressoides* (West in Vahl) Ag., *C. sertularioides* (Gmel), *C. mexicana* Sond ex Kutz and *C. sertrulata* (Forssk) J. Ag. were examined by capillary gas

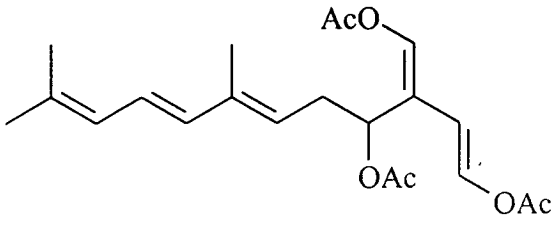
chromatography.[56] The major constituents were palmitic, α-linolenic and hexadecatrienoic acids. The low concentration of C_{16} and C_{18} tetranoic acids were characteristic of this genus. *Caulerpa* species were found to differ from other species by having low C_{18} monounsaturated acids content and approximately equal ω9 and ω7 isomers.[56]

Five species of the genus *Caulerpa* were investigated from the coastal waters near Karachi (Pakistan). *C. chemnitzia* (Esper) Lamour., *C. faridii* Nizam., *C. manorensis* Nizam., *C. racemosa* (Forssk.) J. Ag., and *C. taxifolia* (Vahl) C. Ag. were collected, air-dried at 25^{0}C for one month and analysed for fatty acid, sterol and diterpene constituents.

Palmitic and myristoleic acids were found as major fatty acids, cholesterol was the most abundant sterol and two acyclic diterpene alcohols were reported.[57]

Udoteaceae

Investigations into the green algae from the family Udoteaceae have resulted in the isolation of many metabolites closely related to, or identical with, those found in *Caulerpa* species. The first of these compounds were the sesquiterpenoids rhipocephalin **(57)** and rhipocephanal **(58)** isolated from the Caribbean alga *Rhipocephalus phoenix*.[58] Rhipocephalin is closely related to caulerpenyne **(17)** however it lacks the acetylenic moiety of the latter compound.



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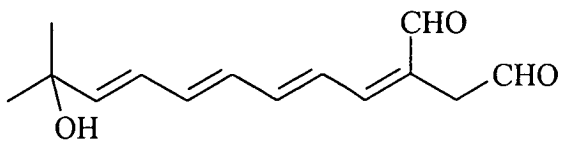
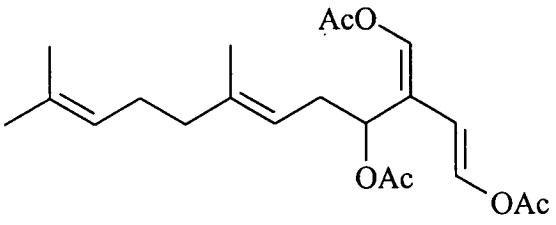
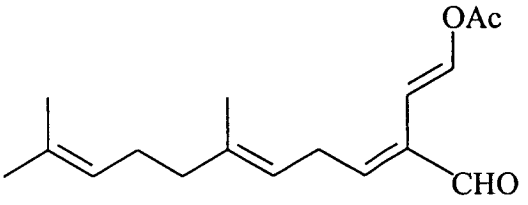


(58)

Flexilin **(15)** isolated from *C. flexilis* has also been identified in two species of the genus *Udotea; U. conglutinata* (Caribbean)[59] and *U. geppii* (Western Pacific).[49] Two similar sesquiterpenoids **(59)** and **(60)** have been isolated from *U. cyanthfomis, Penicillus capitatus* and *Rhipocephalus phoenix*.[37,59]

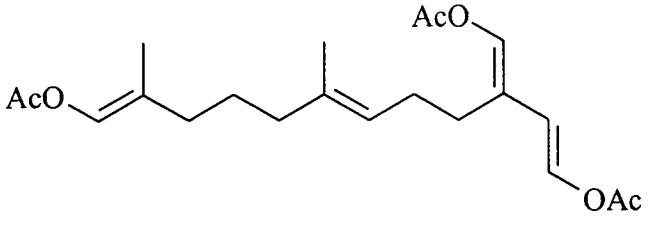


(59)



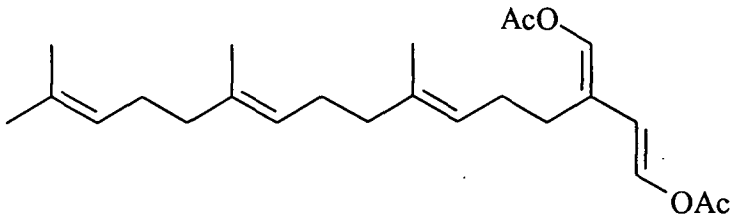
(60)

P. capitatus also yielded the sesquiterpenoid **(61)** which contains three enol acetate functionalities. Despite *Penicillus* being a very common Caribbean algal genus, its natural products were not reported until 1984.[59] This is due to the extreme instability of the secondary metabolites which are quickly degraded by powerful oxidative enzymes on storage. Immediately extracting the alga and liberating the metabolites by chromatography was found to solve this problem.

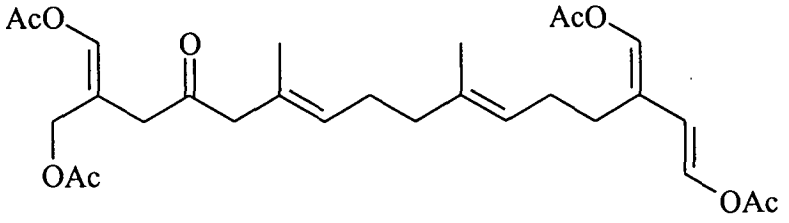


(61)

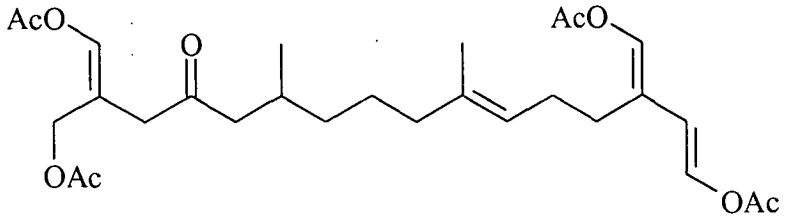
Diterpenoid metabolites have also been identified from algae within the family Udoteaceae. Australian collections of *Chlorodesmis fastigiata* were found to contain didehydrotrifarin **(62)**, chlorodesmin **(63)** and dihydrochlorodesmin **(64)**[59] and the diterpene **(65)**.[60]



(62)



(63)



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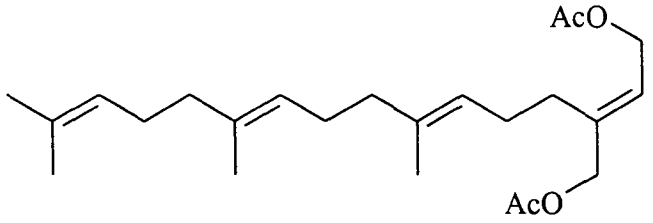
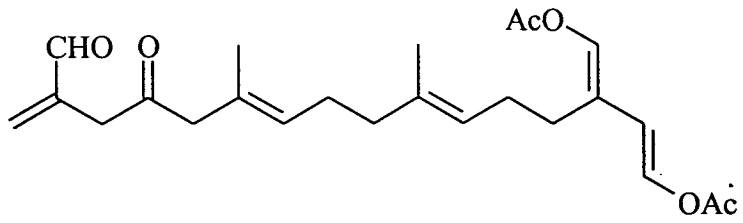
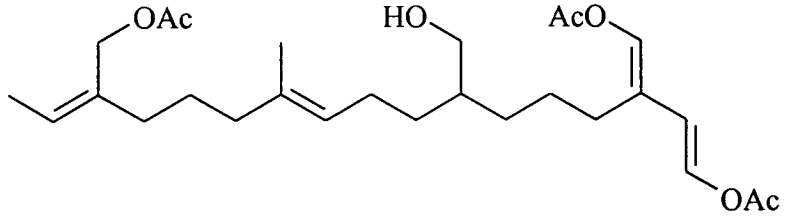


(65)

The compounds **(63)** and **(64)** are unusual in possessing ketone moieties and enol acetate groups on both ends of the diterpenoid skeleton. Chlorodesmin **(63)** has also been isolated from a Guam collection of *C. fastigiata* as was the aldehyde **(66)**.[49] A related diterpenoid **(67)** was isolated from the tropical Pacific alga *Tydemania expeditionis*.[49]

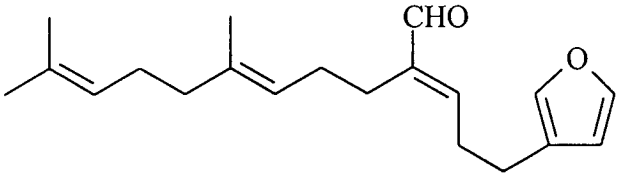


(66)

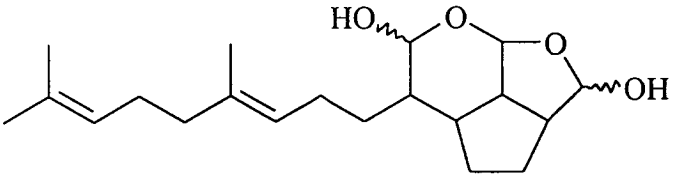


(67)

Udotea is found worldwide inhabiting sandy areas in warmer tropical and sub-tropical areas. Collections of *Udotea flabellum* stored in solvent yielded udoteafuran **(68)** and a complex mixture of hydrates of udoteatrial **(69)**.[61] Similar studies of fresh algae indicate that udoteatrial is generated on storage. The stereochemistry at C-7 of udoteatrial **(69)** has recently been revised following a total synthesis.[61]

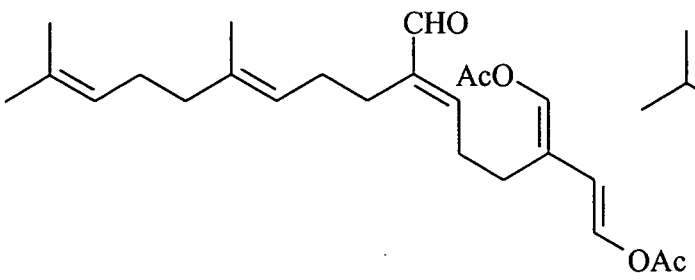


(68)

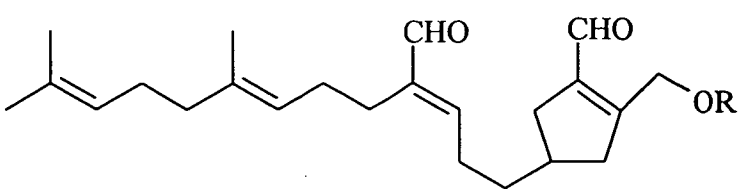


(69)

Udoteal **(70)** was the major metabolite isolated from several species of freshly collected *Udotea* from different locations; *U. flabellum* (Caribbean Sea),[61] *U. argentea* (western Pacific)[75] and *U. petiolata* (Mediterranean Sea).[61] Minor metabolites of fresh *U. flabellum* incude the diadehyde petiodial **(71)** and the alcohol **(72)**.[61]

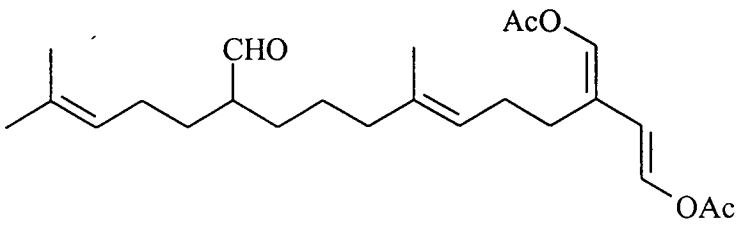


(70)



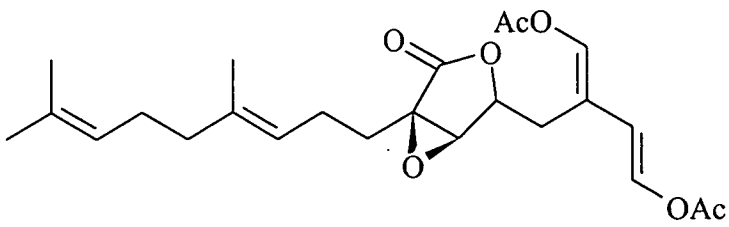
(71) R=Ac (72) R=H

Petiodial (**71**) has also been isolated from *U. petiolata* from the Mediterranean Sea.[61] A diterpenoid (**73**) which is isomeric with udoteal (**70**) has been isolated from *U. spinulosa* collections from the Caribbean.[37]



(73)

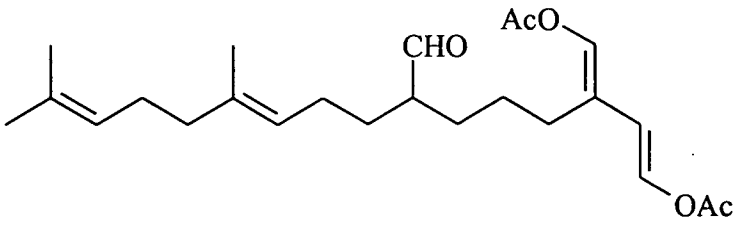
The α,β-epoxyactone (**74**) was isolated from the alga *U. argentea* from Guam.[49]



(74)

Penicillus

Caribbean *Penicillus* species have elaborated related diterpenoids. *P. dumetosus* and *P. pyriformis* both yielded dihyroudoteal (**75**).[62] The related aldehyde (**76**) was also isolated from *P. pyriformis* and the diterpenoids (**77**) and (**78**) were found as minor metabolites in *P. dumetosus*.[61] The triacetate (**79**) was isolated from different collections of *P. dumetosus*.



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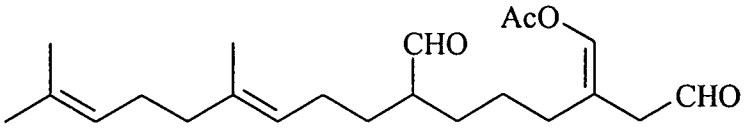

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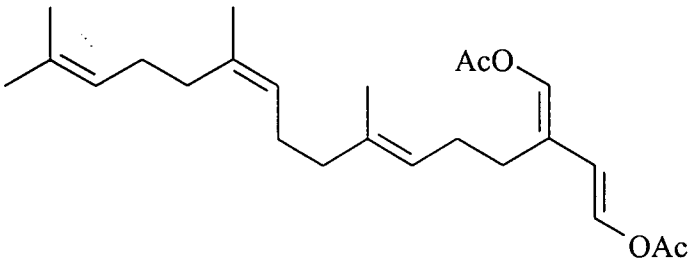

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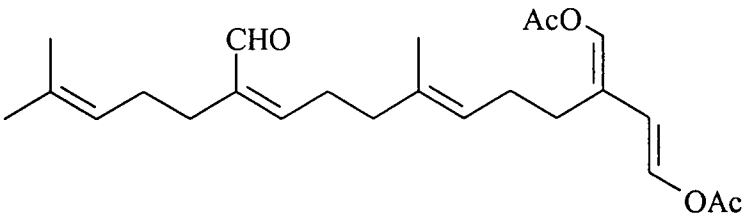

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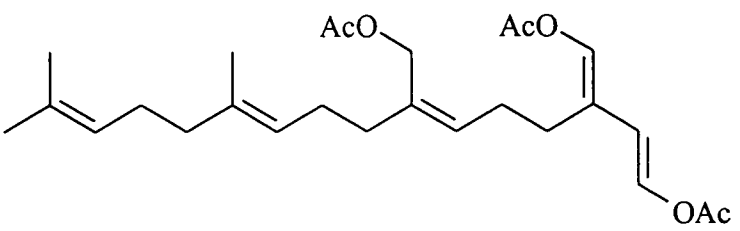
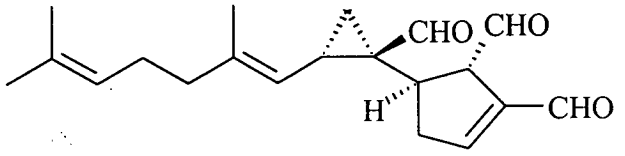


(79)

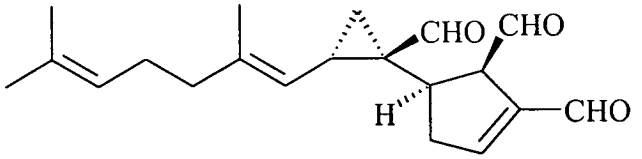
Halimeda

The genus *Halimeda* is the most chemically and biologically interesting of the family Udoteaceae. Halimedatrial **(80)** which contains both a cyclopropane moiety and a trialdehyde functionality has been isolated from many species of this genus.

Epihalimedatrial **(81)** has been found in several *Halimeda* species. This epimer of halimedatrial is readily hydrated and highly unstable, indicating the importance of steroechemistry at the central aldehyde functionality.[63]

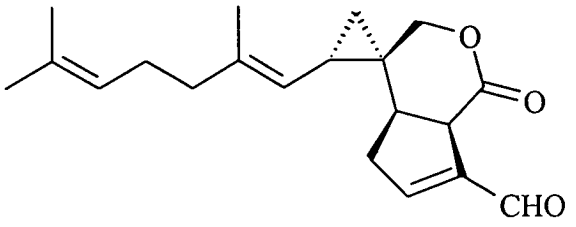


(80)



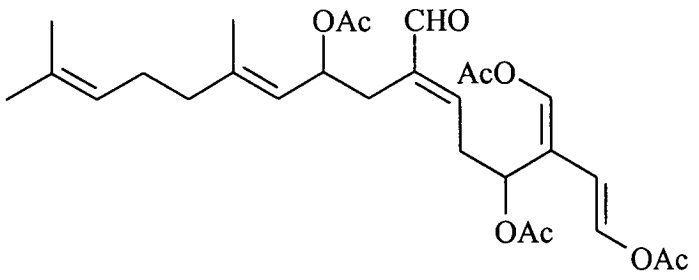
(81)

A related compound **(82)** has been isolated as a minor metabolite from several *Halimeda* species.[64] This compound is a formal redox isomer of epihalimedatrial.



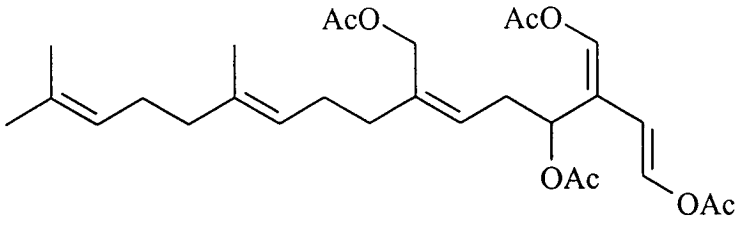
(82)

Most of the *Halimeda* species contain the tetraacetate **(83)**[63] as a major metabolite. This metabolite was first reported from *H. opuntia* (Puerto Rico) but has subsequently been found in nine other Pacific and Caribbean species of the *Halimeda* genus.[63]



(83)

A related metabolite **(84)** has been isolated from *H. goreauii*.[5] The unusual bis-nor diterpenoid **(85)** has been reported as a minor metabolite from several species of *Halimeda*.[63] Compounds **(82)**-**(85)** exhibit antimicrobial activity and are cytotoxic.[64]



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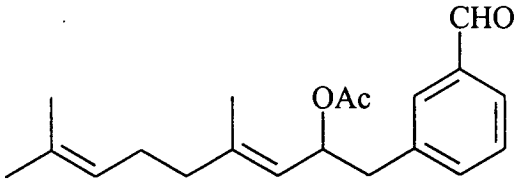
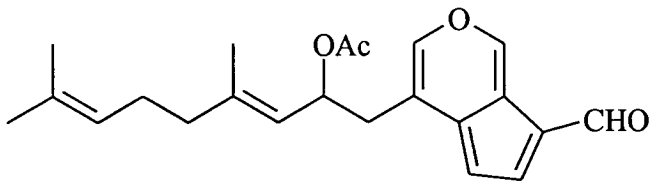


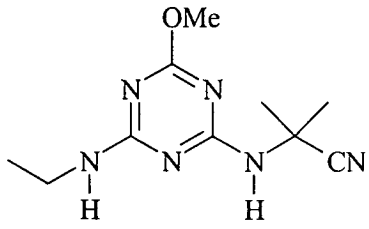
(85)

A novel diterpene aldehyde halitunal **(86)** has been isolated from a Bahamas collection of *H. tuna.* Halitunal showed antivral activity against the murine coronavirus A59 *in vitro.*[65]



(86)

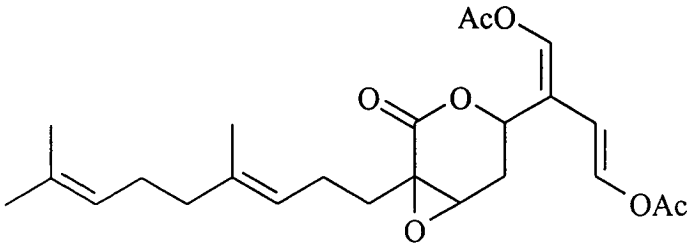
The novel metabolite **(126)** was isolated from *H. xishaensis* collected from the Xisha islands in the South China Sea. The structure of **(126)** was determined by X-ray analysis.[85]



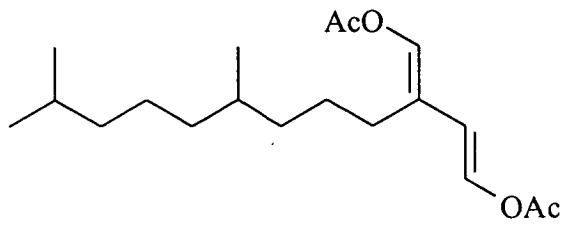
(126)

Pseudochlorodesmis furcellata

Studies of this alga from Guam have resulted in the isolation of two new diterpenoids; the epoxylactone **(87)** as the major metabolite and the acyclic terpenoid **(88)** as the minor metabolite.[66]



(87)

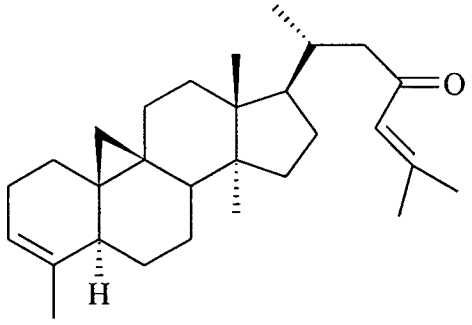


(88)

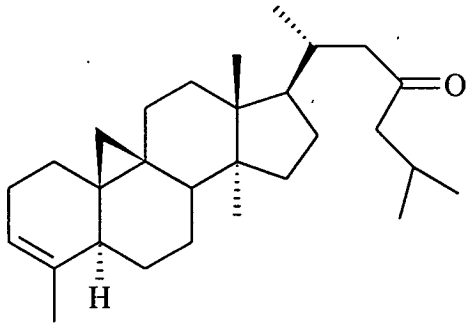
The epoxylactone **(87)** is structurally related to the epoxylactone **(62)**, isolated from *Udotea argentea.*[49] The stereochemistry of the substituents on the lactone ring could only be suggested on the basis of NMR studies and CD experiments.[66]

Tydemania expeditionis

Studies of this alga from the Indo-Pacific have shown interesting results. Collections from Guam and Palau were found to contain three related norcycloartane triterpenoids **(89)-(91).**[66] X-ray analysis was used to determine the structure of triterpenoid **(90)** and the derivatives were intra-converted through oxidation and hydrogenation. Not all collections of *T. expeditionis* contained these triterpenoids, most containing the more typical bis-enol acetate **(67).**



(89)



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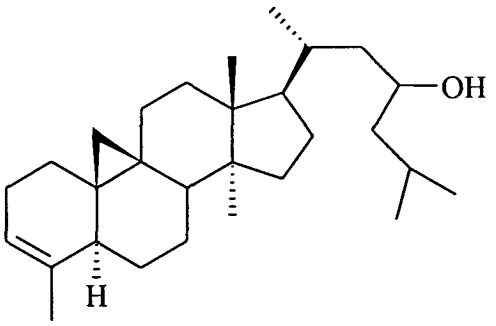
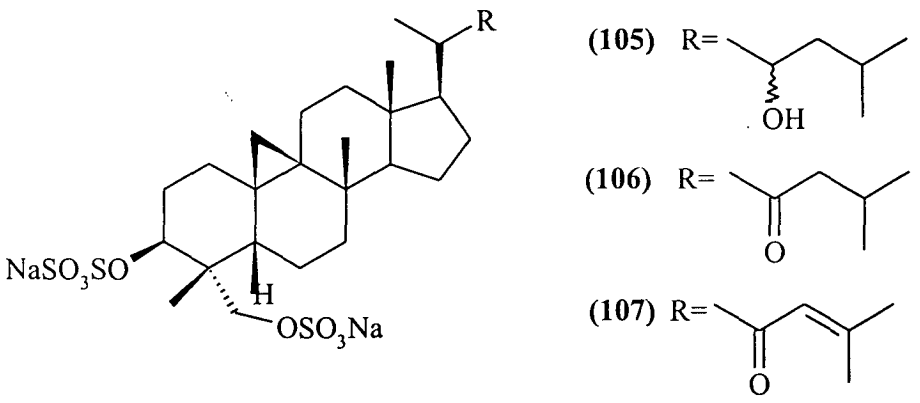


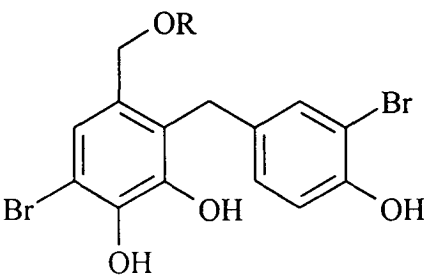
(91)

Further studies demonstrated that extracts of *Tydemania expeditionis* inhibited the oncogenic enzyme tyrosine kinase.[72] A bioassay-guided fractionation of extracts resulted in the isolation and structure elucidation of compounds **(105)-(107)**.



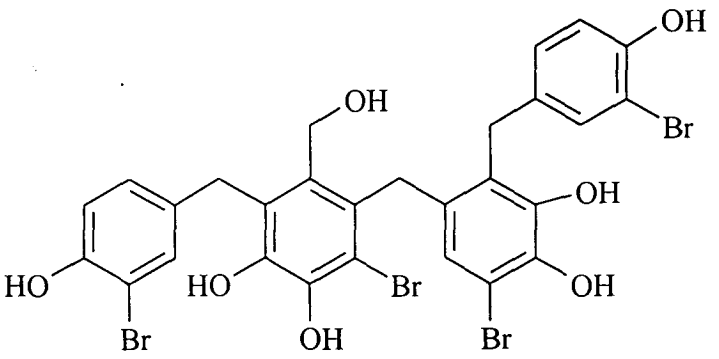
Avrainvillea

Several green algae of this genus produce compounds which are not terpenoid. For example, the bromine-containing diphenylmethane, avrainvilleol **(92)** has been isolated from Caribbean collections of *A. longicaulis*[67] and also from *A. rawsonii* from the Bahama Islands.[37]

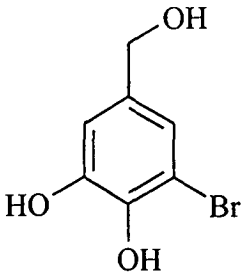


(92) R=H **(94)** R=Me

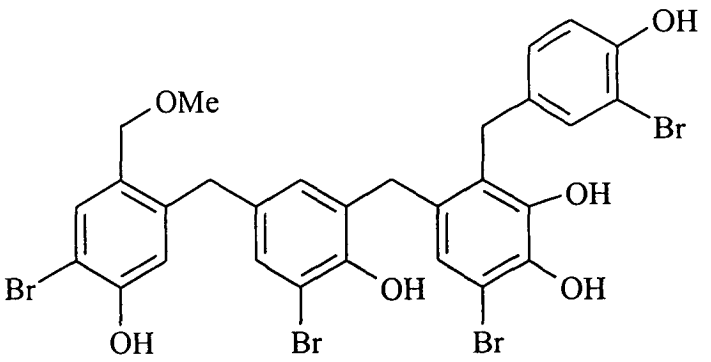
Avrainvilleol **(92)** appears to be produced by dimerization of a *p*-hydroxybenzyl alcohol precursor followed by bromination of the activated phenols. The related alga *Cladocephalus scoparius* from the Bahama Islands produced no unusual natural products.[37] By using an enzyme assay a new compound **(93)** has been isolated from of the active fraction of *A. rawsonii* together with the known compounds **(92)**, **(94)-(96)**.[68] Metabolites **(92)**, **(93)** and **(96)** were active in the enzyme assay. Compound **(92)** has also been reported as having antimicrobial and antifeedant activity whilst **(96)** has been found to be an inhibitor of HMGg-CoA reductase.[68]



(93)



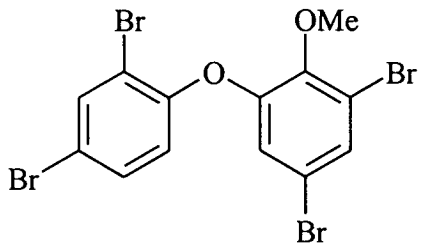
(95)



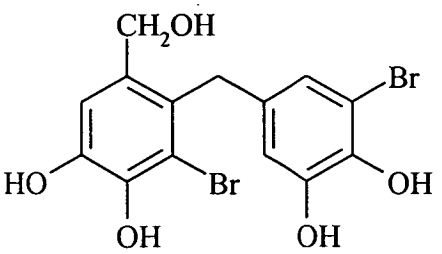
(96)

The brominated metabolite **(97)** which resembles a class of sponge metabolites[69] was isolated from *Cladophora fascicularis* whilst the tropical green alga *A. nigircans*

elaborated the novel metabolite **(98)** as well as the known avrainvilleol **(90)** and 3-bromo-4, 5-dihydroxybenzyl alcohol.[70]



(97)

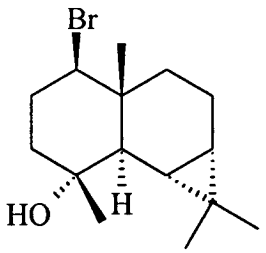


(98)

On the basis of the differing metabolites found in *Avrainvillea* and *Tydemania* these two species should be considered unique members of the Order Caulerpales. It may be significant taxonomically that these two genera produce compounds other than the terpenoid enol acetates and aldehydes characteristic of caulerpean algae.

Neomeris annulata

Halogenated compounds are uncommon in green algae with the first halogenated sesquiterpenes **(99)-(101)** being isolated from a Bermuda collection of *Neomeris annulata* (Dasycladaceae). The metabolites **(99)** and **(100)** were found to be toxic to *Artemia salina* (brine shrimp) and **(101)** showed phytotoxicity towards johnson grass.[71]



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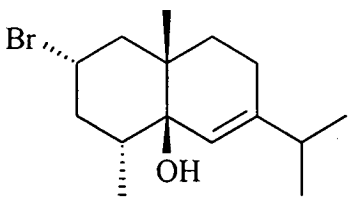
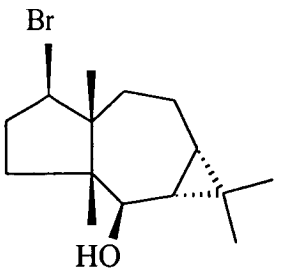
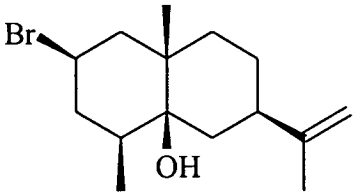


(100)



(101)

A Pacific collection of *N. annulata* yielded the brominated compounds **(102)-(104)**. The compounds **(99), (100), (102)** and **(103)** deterred feeding by herbivorous fishes.[71]



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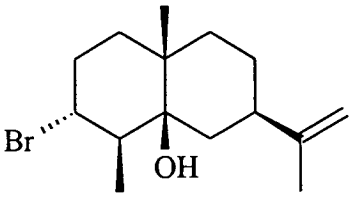

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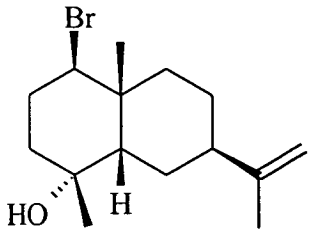
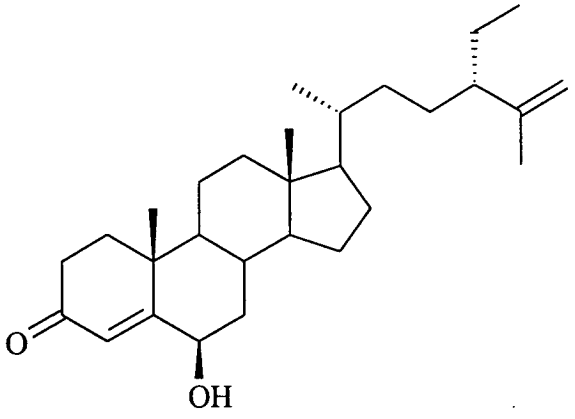


(104)

Codium arabicum

Extracts of this alga exhibited cytotoxic activity against tumour cell cultures. Fractionation of the extracts led to the novel active compounds **(108)-(110)** and the known compounds **(111)-(113)**.[75]



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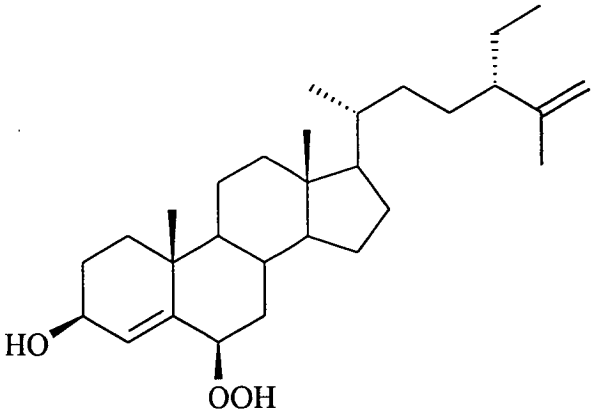

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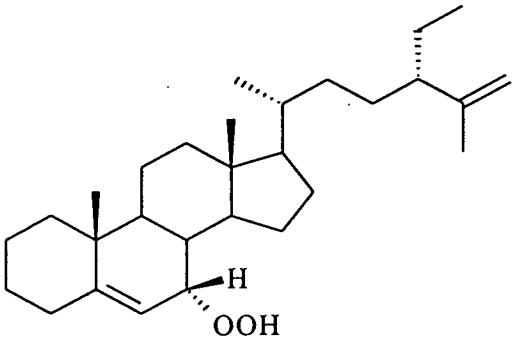

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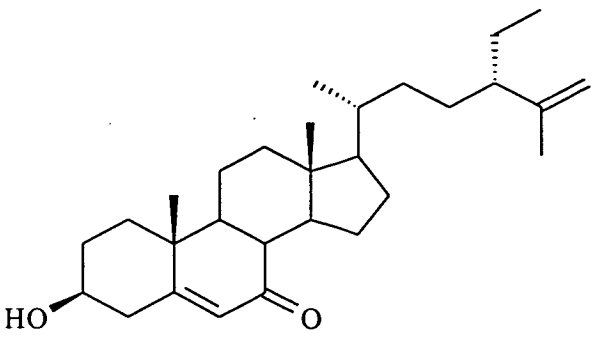

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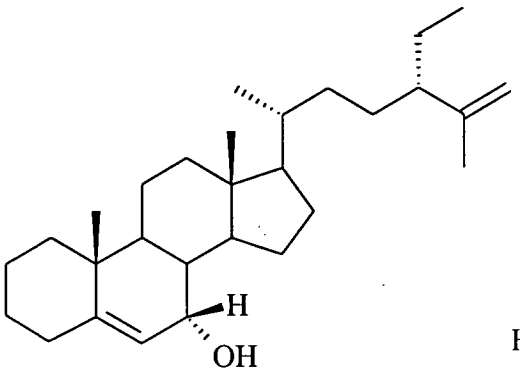

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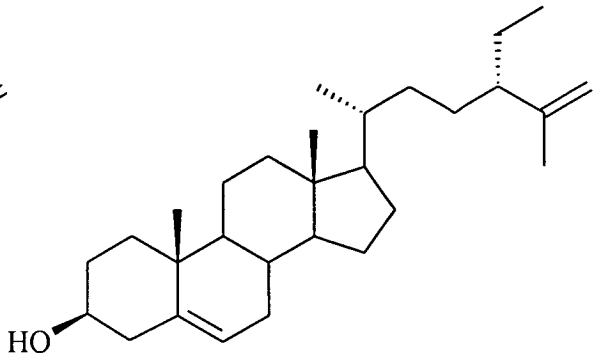
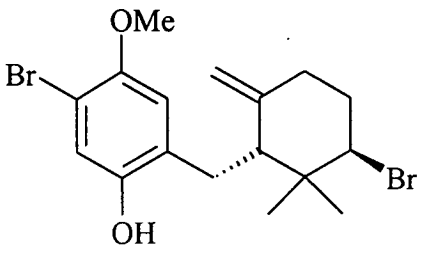


(113)

Cymopolia barbata

This alga produces brominated monoterpenehydroquinones; cymopol and the chromanols.[74] The two diastereomeric compounds 3(*R*)-cyclomopol monomethyl ether **(114)** and its *S*-epimer **(115)** have been obtained in a fractionation of an extract of *C. barbata* guided by a co-transfection assay.[74] Both compounds interact with the human progesterone receptor (hPR).



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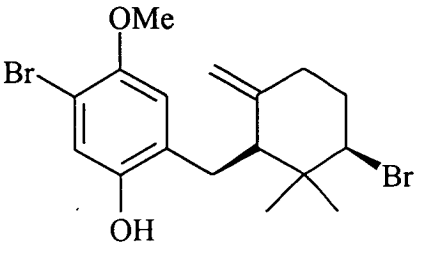
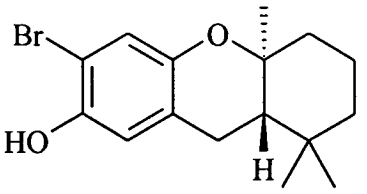


(115)

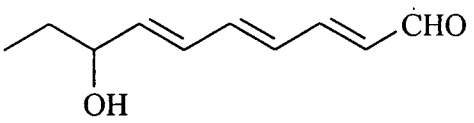
A new chromanol **(116)** was obtained as a minor constituent of *C. barbata*. This compound incorporated into palatable seagrass or alga significantly reduced feeding by the pinfish *Lagodon rhomboides* and the marine amphypod *Hyale macrodactyla*.[74]



(116)

Acrosiphonia coalita

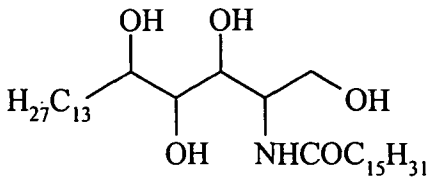
A novel C_{10} conjugated trienal **(117)** has been obtained from the green alga *A. coalita*. In antimicrobial assays **(117)** showed activity against the yeast *Candida albicans* at 100μg/disk.[68]



(117)

Ulva fasciata

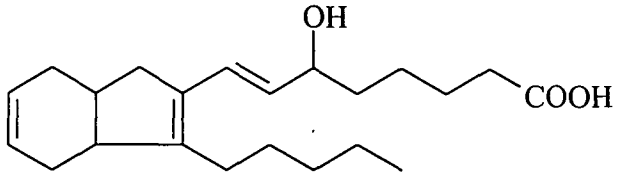
The ethanolic extract of this green alga possessed antiviral activity against the Simeliki Forest virus. The bioassay guided fractionation of the extract resulted in the isolation of the novel compound **(118)** which showed antiviral activity both *in vitro* and *in vivo*.[68] This is the first report of a 2-amido-1,3,4,5-tetrahydroxyoctadecane in a marine species.



(118)

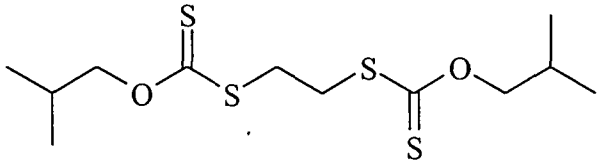
Dictyosphaeria

The novel bicyclic lipid **(119)** has been isolated from the southern Australian green alga *Dictyosphaeria sericea.*[75]



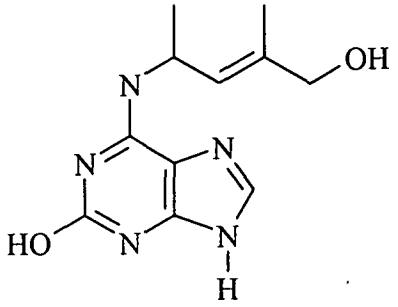
(119)

A bis-xanthate **(120)** was isolated from *Dictyosphaeria favulosa* and identified by X-ray analysis.[76]



(120)

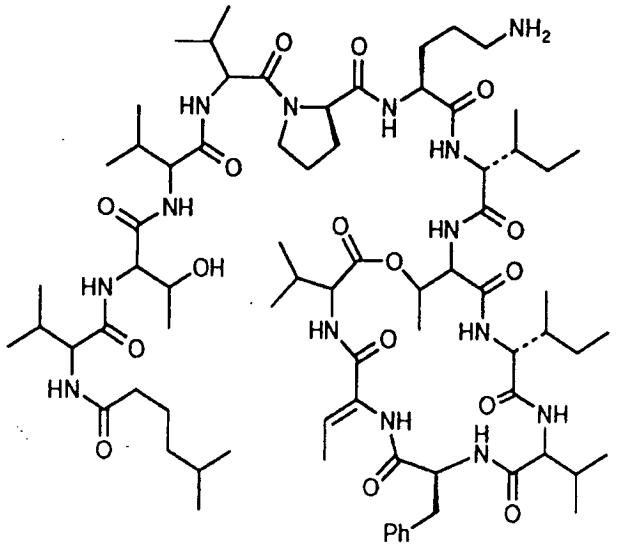
An unidentified green alga from the Goa region of India yielded the cytokyrin **(121)**.[77]



(121)

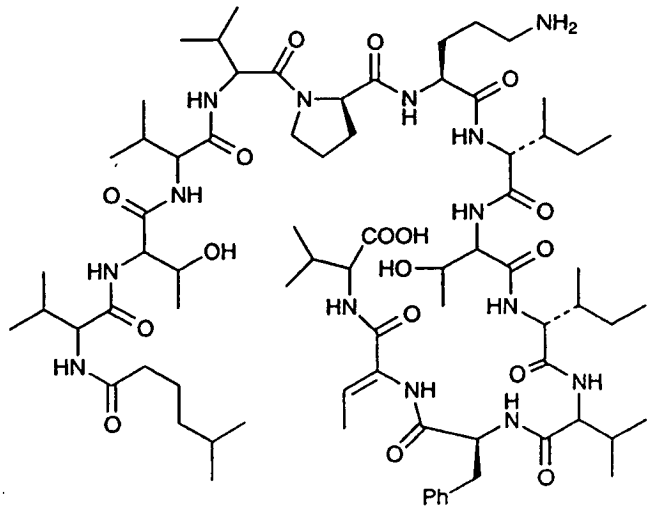
Bryopsis

A Hawaiian species of *Bryopsis* elaborated kahalalide F **(122)** which was also isolated from the sacoglassan *Elysia rufescens* which feeds upon the *Bryopsis* species.[77]

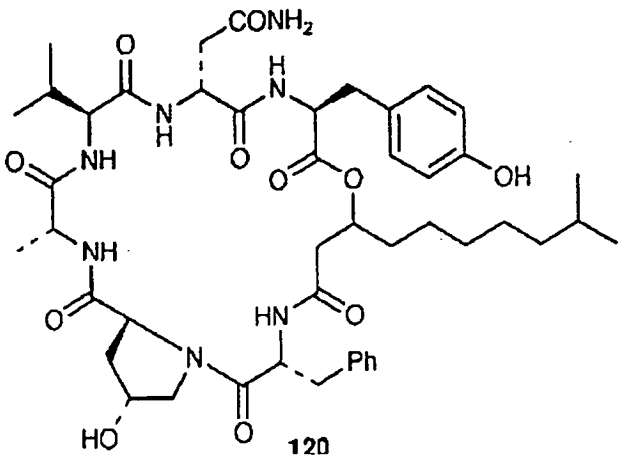


(122)

Kahalalide F **(122)** was found to have antiviral, antifungal and cytotoxic properties. A subsequent investigation of the same alga elaborated kahalalides A, B, F, G and K. Only kahalalide G **(123)** and K **(124)** were unique to the *Bryopsis* species.[78,79]

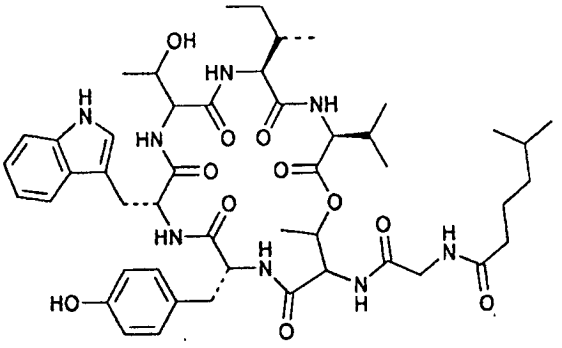


(123)



(124)

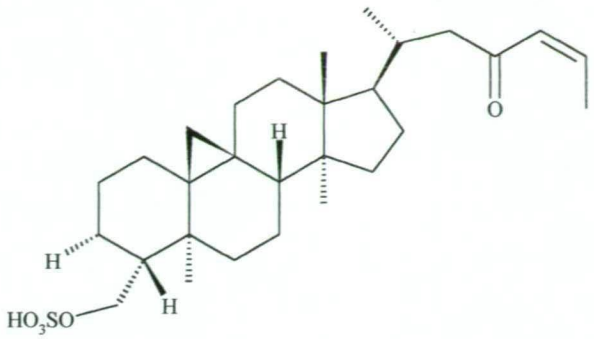
An additional depsipeptide kahalalidide O **(125)** was found more recently in both the green alga *Bryopsis* species from Oahu and the sacoglassan mollusc *Elysia ornata* that feeds upon it.[80]



(125)

Tuemoya sp.

A novel cycloartenol sulfate **(127)** has been isolated from a *Tuemoya* sp. from an unidentified location. This metabolite was found to be an inhibitor of VZV and CMV proteases.[81]



(127)

It can be seen from the preceding review of the literature on green algal metabolites that the metabolites isolated comprise a variety of structural types and biological activities. However the number of metabolites isolated from the order Caulerpales comprise more than 60 % of the green algal metabolites discussed in this section. These metabolites almost without exception contain aldehyde or acetoxy moieties in varying configurations and possess biological activities chiefly attributed to these structural components. The metabolites isolated from the remaining orders of green algae consist of approximately 8 % halogenated metabolites and 32 % of metabolites with more than a dozen structural types (Chart 1.1).

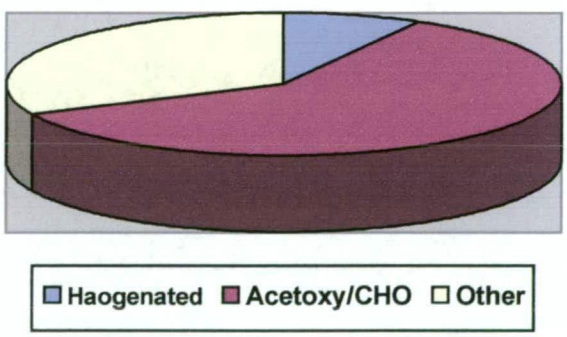


Chart 1.1 Variation of marine green algal metabolites

1.3 Macroalgae in southern temperate waters

The region encompassing southern temperate waters includes the whole southern coast of Australia (including Tasmania). The western limit is defined as Cape Naturaliste in Western Australia (latitude 27^0) and the Eastern limit as the Victoria-New South Wales border. This region is known as the Flindersian biogeographic province (Figure 1.2). A biogeographic province is defined as a coastal region characterised by a relatively distinct and homogenous flora and fauna with only a small percentage of species common to adjacent provinces, and usually differing in temperatures from adjacent provinces by more than 5 degrees Celcius.[82]

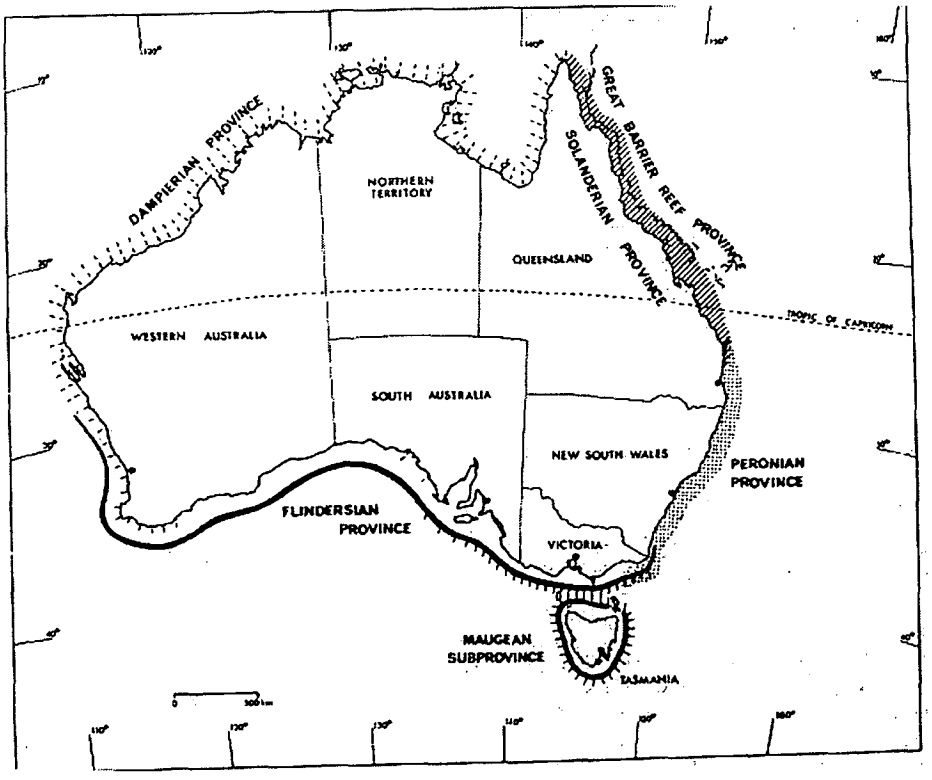


Figure 1.2 Map of Australia showing the biogeographic provinces[82]

The marine environment of southern temperate Australia contains significant levels of marine biodiversity and endemism, with the biodiversity of the marine macroflora being amongst the highest in the world (Figure 1.3). While other regions have more species, their coastal waters cover a wider range of climates.[85]

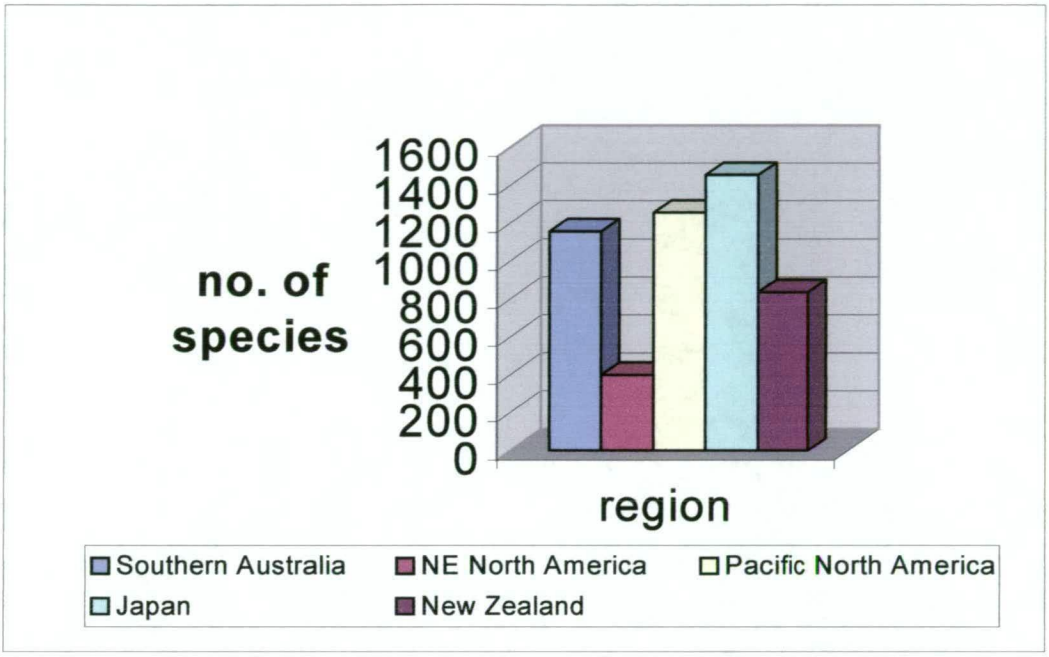


Figure 1.3 Global macroalgal biodiversity (number of species vs. region)

Within Australia the biodiversity of temperate species of macroalgae is three times that of tropical species;[86] for example of the 685 genera (4000 species) of red algae world wide 43 % of genera and 20 % of species occur in southern Australia. Over 75 % of red algae, 57 % of brown algae and 30 % of green algae do not occur outside this region[86] with more than 100 species still to be named.

The Tasmanian algal flora remains poorly known due to the lack of professional algal researchers, however the south-eastern Tasmanian region includes probably the highest level of localised endemic species in Australia.[87] The shallow reefs around eastern, southern and western Tasmania are more thickly vegetated with macroalgae than

reefs elswhere in Tasmania presumably due to the cool water temperatures and the relatively higher nutrient levels.[88] Many of the recorded species of macroalgae in Tasmania also extend along the southern Australian coast and into South Australian and southern Western Australian waters (Flindersean province). In this province 83 % of the known macroalgal species occur in Victoria, Tasmania and eastern South Australia; 60 % of species occur west of Kangaroo Island and 45 % of species along the entire coast.

The long period of geological isolation of this region (65 million years) compared to tropical Australia (20 million years) has led to high levels of endemism whilst the low level of nutrients in Australian coastal waters has promoted biological diversity and evolutionary strategies that rapidly harvest, use and recycle the available nutrients. Other factors which have contributed to the region's biodiversity and endemism are the extensive continental shelf, the long east-west coastline and the ice-free extent of the south facing coastline.[85]

The drowning of Bass Strait and the effects of winds, seas, swells and tides on Tasmania have created a number of different marine communities. The Bass Strait islands, the North, North-east, East and West coasts all have markedly different marine habitats due to the different physical conditions experienced on each coastline.

The Tasmanian and Victorian coasts are classified as cold temperate whilst South Australia and the southern coast of Western Australia are intermediate between cold and warm temperate. The range of summer and winter temperatures for the southern region are shown in Figure 1.4.

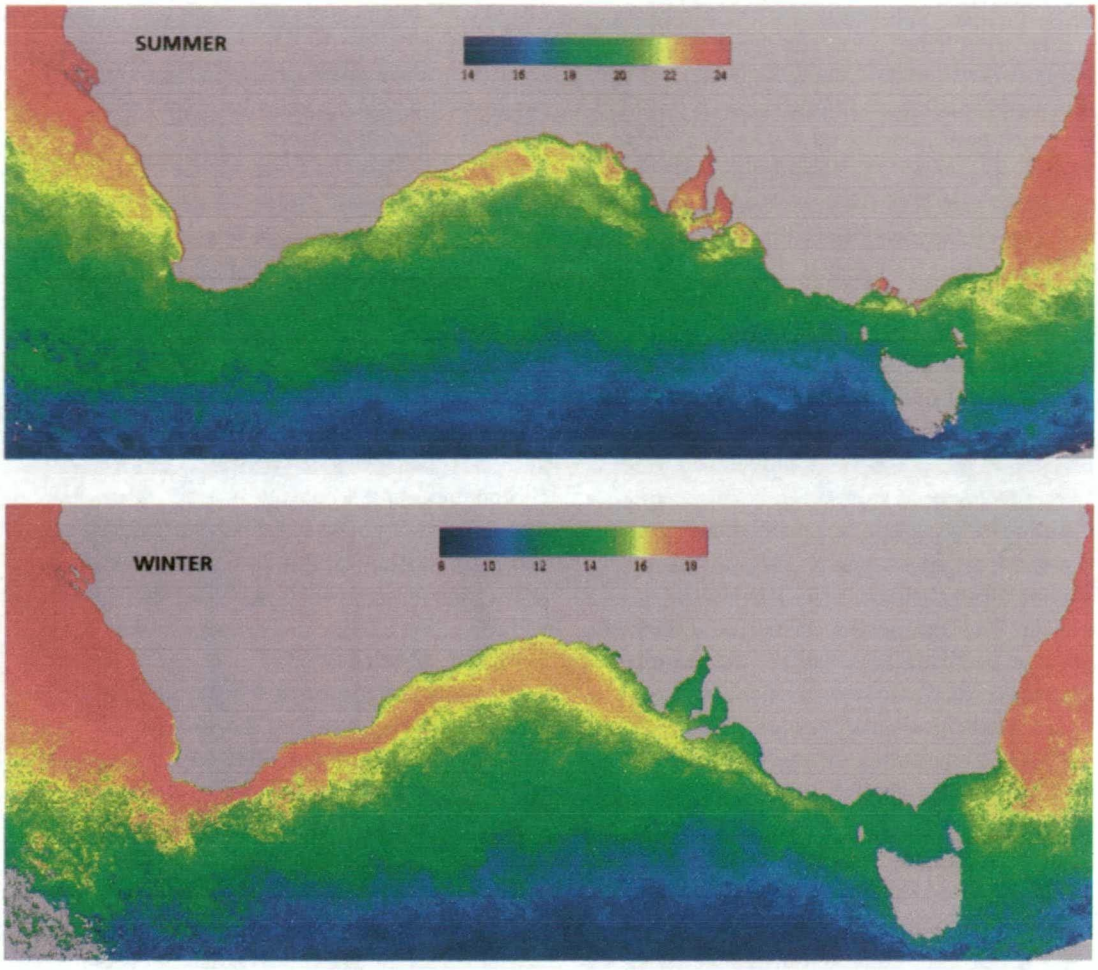


Figure1.4 Temperature variation (^{0}C) for southern Australian waters[85]

The following inter-tidal zones are recognised on most coastlines of the world (Figure 1.5):

The **littoral fringe** which is wetted only by extreme high tides, the **eulittoral zone** which consists of most of the intertidal zone; (above this zone there is only irregular wetting and below this zone the organisms are immersed) and the **sub-littoral zone** which is normally immersed; (most rocky coasts and rough water coasts). The **eulittoral zone** is further divided into **upper, mid** and **lower**. A feature of most temperate Australian coasts is the lack of conspicuous algae above the **lower eulittoral zone**, at least during summer.

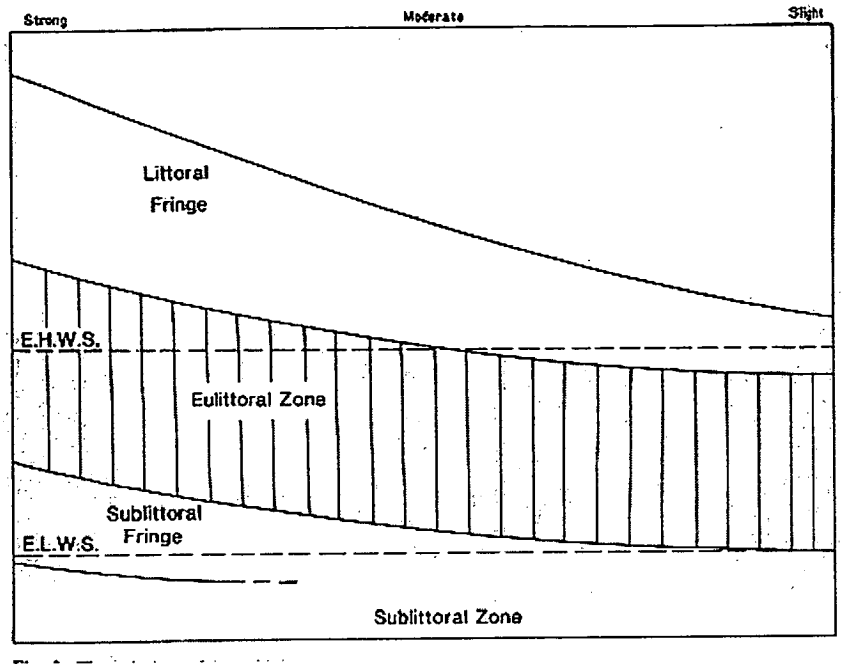


Figure 1.5 The relation of intertidal zones to tidal level and water movement. (E.H/L.W.S – extreme high/low water spring tides).[84]

1.4 Dominant organisms on southern Australian coasts

On rough water coasts the littoral fringe is dominated by littorinid snails with occasional black patches of the blue- green algae *Calothrix fasciculata* or *Entophysalis deusta* above the littorinids. Lichens occur above the littoral fringe on Tasmanian coasts. The green alga *Prasiola* occurs several metres above high tide level in southern Tasmania and off the Victorian coast. In the eulittoral zone animals dominate the upper two thirds with the lower third covered by an algal mat whilst in the sublittoral zone the larger brown algae dominate, generally with a sharply defined upper boundary.

Three algal-dominated subtidal zones are recognised on rocky coasts (upper, mid and lower sublittoral zones). Chlorophyta are prominent in the upper sublittoral zone, with some extending above low water level. Several species of *Caulerpa*, *Bryopsis, Ulva, Enteromorpha, Cladophora, Chaetomorpha* are common.

On moderately sheltered to calm-water coasts in the littoral zone the rock is relatively bare and the more usual substrate is sandy mud, bearing samphires (*Sarcocornia* and *Arthrocmum*). In the eulittoral zone barnacles, red algae, molluscs, blue-green and coralline algae, beds of the seagrass *Zostera* and the green algae *Ulva, Enteromorpha,* and *Chaetomorpha billardierii* predominate. The sublittoral zone is dominated by large brown algae with an understorey of coralline algae and often species of *Caulerpa.* Seagrasses cover extensive areas on the sandy bottom below rocky areas and become more extensive on sandy mud tidal flats. Only a few algae can tolerate the lack of water movement on the mud flats.[82]

1.5 Tasmanian marine green algae

Approximately 140 species of benthic (attached) green algae are known from southern Australian waters with about 60 % being confined to the region. Two of the families of the order Caulerpales (Udoteaceae and Caulerpaceae) are represented on southern Australian coasts. Of the family Caulerpaceae which contains the single genus *Caulerpa*, nineteen species of *Caulerpa* are found, with fifteen of these species being endemic. The slightly cooler south-eastern coasts are richest in such species with many attaining their largest size in this area. Species of *Caulerpa* grow vegetatively from a horizontal stolon and therefore can rapidly colonise large areas of substrate. In terms of abundance and number of species they are often the dominant plants on reef and sand habitats in southern Australia.[82]

The Blackman research group has collected marine organisms over the past twenty-five years, by SCUBA diving in areas close to the shore. Figure 1.6 shows the number of different *Caulerpa* species collected at our collection sites prior to 1996.

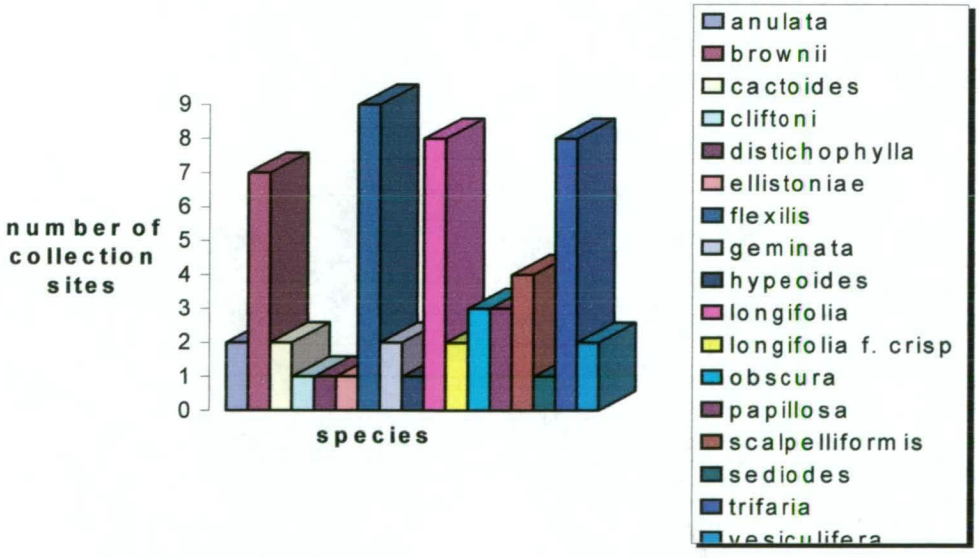


Figure 1.6 Tasmanian collections of *Caulerpa* species (prior to 1996)

Details of the *Caulerpa* collections undertaken for this thesis, during the period September 1996 to March 2001 are shown below in Table 1.3.

Date of Collection	Location	Species collected	Comments
27 Sept 96, 5 Nov 96	Taroona	*C. trifaria*	-From rocks out to 5 m in depth
16 Nov 98		*C. trifaria. C. longifolia*	-*C. trifaria* abundant, *C. longifolia*
19 Feb 99		*C. trifaria. C. longifolia*	new growth, other green algae in decay
26 May 99		*C. trifaria*	-old plants, many totally decayed
10 Aug 99		*C. trifaria*	-new growth
18 Oct 99		*C. trifaria*	-peak condition, dark green, in large clumps, two holothurian sp. on plants (black/orange)
9 Mar 97, 17 Nov 97	Eagle Hawk Neck	*C. trifaria*	
27 Mar 97	Gordon	*C. trifaria*	
14 Aug 97	Mayfield Bay	*C. trifaria, C. brownii, C. flexilis, C. scalpelliformis*	
19 Nov 97		*C. trifaria*	
26 Oct 98	Bicheno	*C. trifaria*	
6 Sept 99		*C. flexilis*	-jetty, *C. flexilis* on a rock, no other *Caulerpa* sp.
23 Nov 98	Spikey Beach	*C. trifaria, C. brownii* (branched), *C. flexilis, C. longifolia, C. scalpelliformis*	-LHS of large S rock, in the channel, N along rocks to main beach
5 Mar 99		*C. trifaria, C. flexilis, C. longifolia, C. scalpelliformis*	-8 m anticlockwise around large rock beyond beach
28 Jun 00		*C. scalpelliformis*	-directly off beach, 2-4 m depth, abundant, halfway to peak growth
16 Mar 01		*C.scalpelliformis*	-abundant
12 Jan 99	Lady Bay	*C. trifaria, C. brownii* (branched), *C. flexilis, C. trifaria*	-N of carpark, 2-10 m in depth

Date of Collection	Location	Species collected	Comments
May 99	Spring Beach	*C. brownii, C. flexilis, C. longifolia, C. scalpelliformis*	- rocks at the S end, large areas of pure *C. brownii* (unbranched) halfway to Point.
3 Nov 99		*C. brownii, C. flexilis*	- *C. brownii* (branched) peak growth, (unbranched) juvenile stage, *C. flexilis* end of optimum growth.
8 Mar 00		*C. brownii, C. flexilis*	- *C. brownii* (branched), and (unbranched) patches side by side. Large patch *C. brownii* (unbranched) on large rock shelf in 1.5 m depth near the end of the Point, "young" plants. *C. flexilis* 4 m depth, end of Point, only one patch.
1 Sep 00		*C. brownii* (unbranched)	- optimum growth, mostly close to rocks and growing on boulders.
31 Mar 99	Tinderbox	*C. trifaria, C. longifolia*	- *C. trifaria* 100 m "upstream", *C. longifolia* on rocks, 1-2 m depth
7 Jul 00	Shelley Beach	*C. flexilis*	- just before the reef, abundant growth

Table 1.3 Tasmanian *Caulerpa* collections undertaken for this thesis

Figure 1.7 shows the location of these collection sites. The following chapters detail the investigation of five Tasmanian species of *Caulerpa* for their secondary metabolite content. A number of novel secondary metabolites are described from *C. trifaria* (Chapter 3), *C. brownii* (Chapter 4) and *C. flexilis* (Chapter 5). Two *Caulerpa* species; *C. scalpelliformis* and *C. longifolia* (Chapter 6) have been investigated chemically for the first time. Seasonal, geographic and other factors influencing the production of secondary metabolites are also discussed. An outline of the bioassays used to determine the biological activity of extracts and/or compounds is included in Chapter 2.

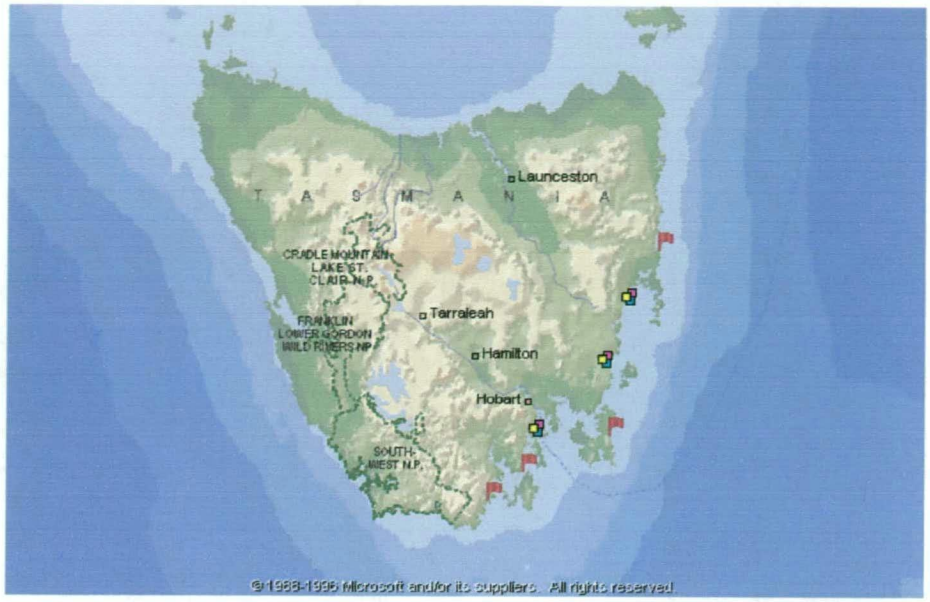


Figure 1.7A Overview of collection sites for Tasmanian *Caulerpa* species

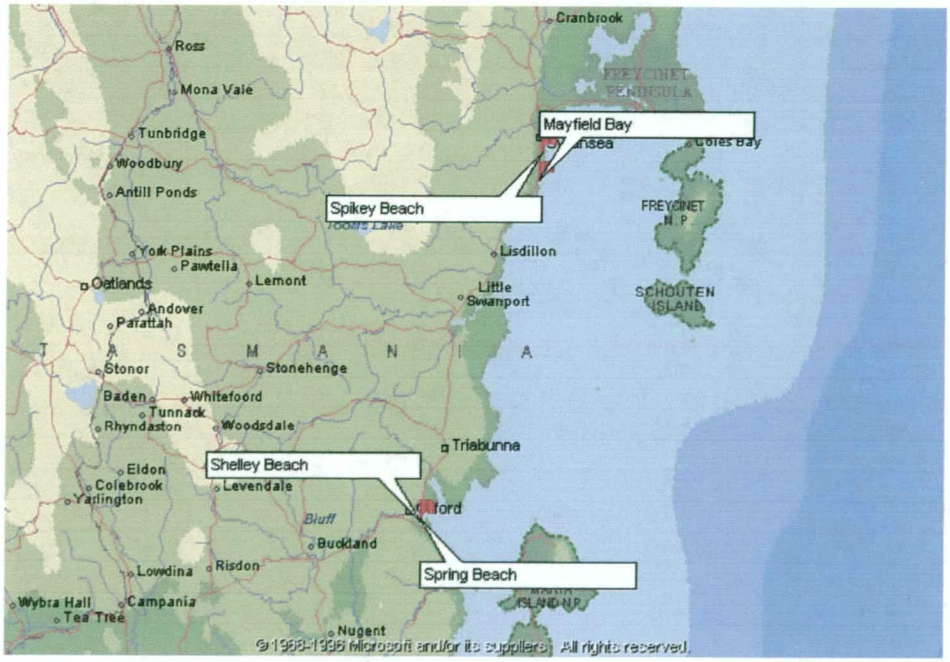


Figure 1.7B Location of eastern collection sites of Tasmanian *Caulerpa* species

Figure 1.7C Location of southern collection sites of Tasmanian *Caulerpa* species

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Chapter

Two

Biological activity of Tasmanian

Caulerpa species

Contents

2.1 Introduction

Bioassays were used during the course of this thesis to gain an insight into the biological activities of both crude *Caulerpa* extracts and also some of the pure secondary metabolites in particular species. The bioassays included the brine shrimp assay and lettuce seedling assay in our own laboratories and a larvae settlement assay used by the Zoology Department at the University of Tasmania. This chapter describes each bioassay and summarises the results obtained for *Caulerpa* extracts and metabolites.

2.2 The brine shrimp (*Artemia Lynch*) assay

The brine shrimp assay was used during this research project due to its reputation as a rapid, reliable, inexpensive and convenient bioassay. This assay was developed by B. N. Meyer and co-workers[1] in 1982 to determine the physiological activity of higher plant extracts and to monitor that activity during the fractionation process.

The eggs of the brine shrimp are obtained from pet shops where they are sold as food for tropical fish. They remain viable for years in the dry state. Within 48 hours of being place in a brine solution, the eggs hatch providing large numbers of larvae (nauplii). Previous bioassay systems that have employed brine shrimp include the analysis of pesticide residues[2], mycotoxins[3], stream pollutants[4], anesthetics[5], dinoflagellate toxins[6], morphine-like compounds[7] and toxicants in marine environments.[8] Usually the hatched nauplii have been used although inhibition of the hatching of the eggs has also been investigated.[9] The method used by Meyer and co-workers involved the testing of the ethanol extracts of seeds of 41 species of Euphorbiaceae; a family of plants which contains toxic compounds of diverse structures. The results[1] showed that this method was a useful assay in determining the activity of plant extracts with eighteen of the euphorb extracts displaying toxicity (LC_{50}< 1000μg/ml).

The procedure used in our laboratory for *Caulerpa* extracts and metabolites, involved 50 μL of solution (extract or pure compound) of concentration 10 mg/mL (in $CHCl_3$) being dispersed on a small disc of filter paper. The solvent was then allowed to evaporate and 5 mL of seawater and 10 brine shrimp were added to the vial. A small sprinkle of dry yeast was then added and the vial placed in a water bath maintained at 27^0C. (Full details of the assay are included in the Experimental section of this thesis).

Five controls and five test vials were produced for each extract/compound. The % deaths were calculated after 24 and 48 hours as (test-control/control) x 100.

The following *Caulerpa* extracts were tested in the brine shrimp assay: *C. trifaria, C. brownii* (branched), *C. brownii* (unbranched), *C. flexilis* and *C. scalpelliformis.*

All extracts showed some level of activity with the order of activity being *C. scalpelliformis* (70%), *C. trifaria* (50%), *C. brownii* (unbranched) (50%), *C. flexilis* (40%) and *C. brownii* (branched) (10%). The LC_{50} values are shown in brackets. There was also a correlation between the amount of secondary metabolites present in the extracts and the degree of activity: extracts tested within a month of collection showed higher activity than the same extracts stored at -20^0C for a period of months. For example *C. trifaria* stored extracts showed lower activity (10-20%) than freshly extracted samples. N.m.r. studies described elsewhere in this thesis consistently show that the concentration of secondary metabolites present in freeze-dried or extracted material decreased on storage.

A small number of *Caulerpa* secondary metabolites were tested using the brine shrimp assay. The novel compounds **(5)** and **(8)** (Chapter 3) from *C. trifaria* and the known toxic secondary metabolite caulerpenyne **(3)** (Chapter 6) from *C. scalpelliformis* were tested and showed high activity; with the dialdehyde **(8)** showing higher activity (70%) than the compounds **(5)** and **(3)** (40%). The 1,4-diacetoxybutadiene and α,β-unsaturated dialdehyde moieties are known for their biological activities; this topic is discussed further throughout this thesis.

2.3 The lettuce seedling bioassay

The lettuce seedling bioassay[10] was employed with one *Caulerpa* species; *C. scalpelliformis*. This assay involved dissolving the crude extract in solvent (0.1g/mL) and testing this solution at varying concentrations on the inhibition of germination and growth rates of lettuce seedlings.

0.1 mL of the *C. scalpelliformis* solution was added to 20 mL of a 1 % solution of agar at 80^0C in a petri dish. After the agar had set, 10 lettuce seeds were added and the dish was covered with a watch-glass and observed over several days (experiment 1). The experiment was repeated with dilutions of $1/10^{th}$ and $1/100^{th}$ concentration of the original solution (experiments 2 and 3 respectively.) Control dishes were also prepared. Two petri dishes were prepared for each concentration. After 7 days the seedlings were removed from the agar and measured.

The results of this assay showed that the lettuce seeds germinated after 5 days in the control dishes, however very little growth was seen in experiment 1 whilst some growth was seen in experiment 2. In experiment 3 the amount of growth was similar to that of the control seedlings. In experiment 1, 50 % of seeds failed to germinate, in experiment 2, 30 % failed to germinate whilst in experiment 3 and the control dishes all seeds germinated. A photo of the seedlings after removal from the agar at the completion of these experiments is shown in Figure 2.1.

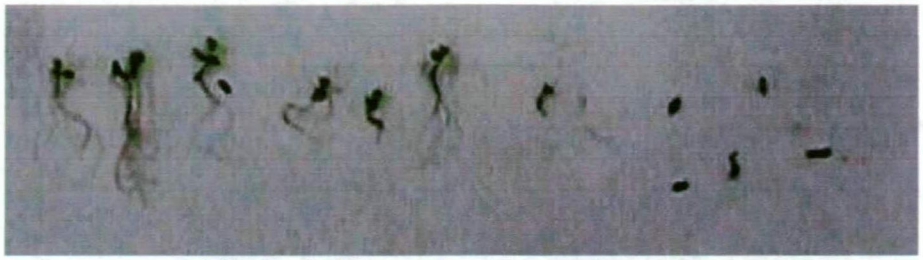

Figure 2.1A results of experiment 1

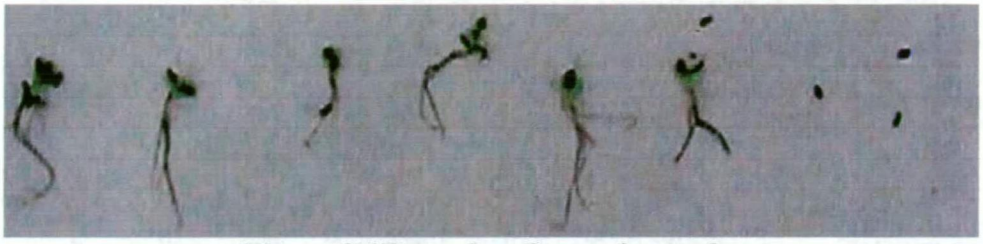

Figure 2.1B results of experiment 2

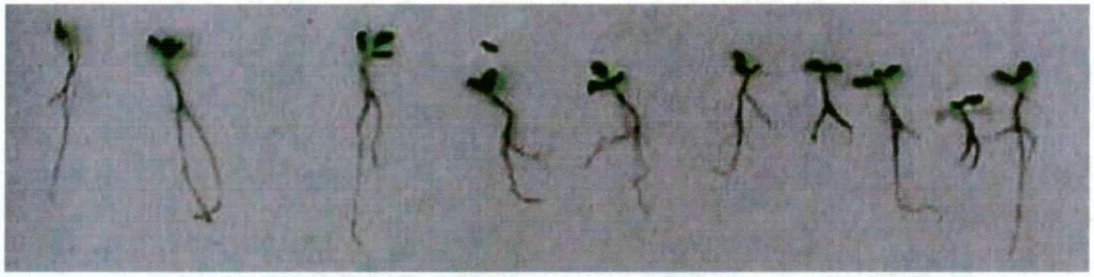

Figure 2.1C results of experiment 3

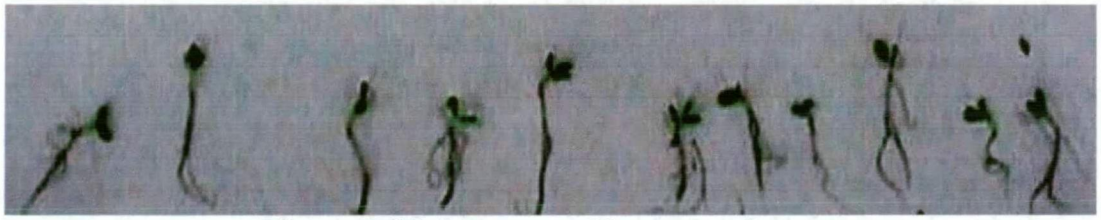

Figure 2.1D lettuce seedling controls

A t.l.c. version of this experiment was also done (experiment 4); where the extract of *C. scalpelliformis* was applied to a t.l.c. plate and developed in 10 % EtOAc in pet. ether as eluent. The solvent was allowed to evaporate and the location of the separate bands marked with a pencil. The plate was then placed in a suitable container and covered with agar solution (2 mm depth). Rows of lettuce seeds were then carefully

placed on the bands and on a control area and the container was covered and observed over several days. Despite cracks in the agar, which made it difficult to make quantitative measurements, the lettuce seedlings on the main band (caulerpenyne) grew less than on the control area whilst a smaller effect was seen on the other bands. Figure 2.2 shows a diagrammatic representation of the results of this experiment.

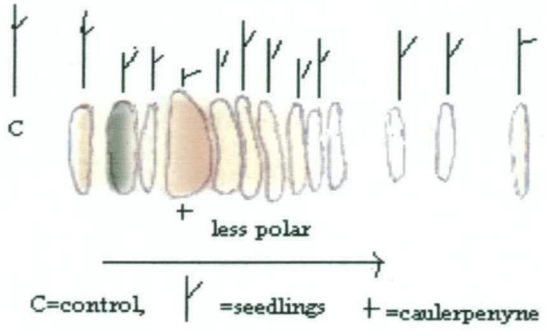

Figure 2.2 Diagram of experiment 4 results

2.4 Larvae settlement bioassay

DCM extracts of three *Caulerpa* were provided to the Zoology Department at the University of Tasmania for use in a study of larval settlement of marine organisms. This study involved the preparation of gel panels impregnated with *Caulerpa* extracts which were then hung on a rope from the uprights of the jetty at Triabunna, on the east coast of Tasmania. Seven panels of each extract and three control panels were used. After 2 weeks the panels were raised and the number of ascidians and bryozoans on the panels were counted. After three weeks all panels were collected.

DCM extracts of *C. scalpelliformis, C. flexilis* and *C. brownii* (unbranched) were provided for testing. The results showed that all three extracts discouraged settling with *C. brownii* (unbranched) being the most active. A description of this experiment is included in the Experimental section of this thesis.

2.5 Conclusions from bioassay testing

The bioassays used in this thesis suggested that all of the *Caulerpa* species studied contained secondary metabolites with some biological activity. Where crude extracts showed low activity, this was generally found to be related to the decomposition of secondary metabolites present in the extract on storage. Since the presence of aldehyde and acetoxy moieties was apparent from the ^{1}H n.m.r. spectra of the extracts and it is known from the literature[11] that such moieties indicate biological activity, more extensive testing of extracts, fractions and pure metabolites was not undertaken. It was decided however to submit pure metabolites to the Norvartis pharmaceutical company for testing against a range of parasites. Due to the curtailment of experimental work as explained in the Acknowledgements section of this thesis, this follow up testing of activity has not yet been completed.

2.6 References

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Chapter Three

Studies of the Tasmanian green alga

Caulerpa trifaria

Contents

3.1 Introduction

C. trifaria is found in waters as far afield as Cottlesloe, W.A., in Western Port, Victoria and around Tasmania.[1] Our research group has collected *C. trifaria* from at least fourteen locations over the past twenty five years. During the course of this thesis twelve collections were made from eight different sites (Chapter 1, Table 1.3).

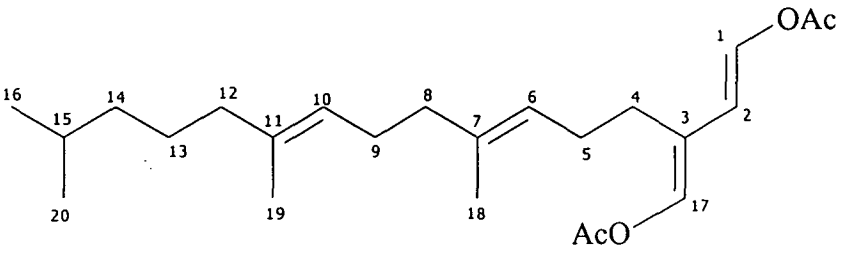
C. trifaria possesses a horizontal, slender stolon attached by rhizoids and erect fronds with simple or occasionally branched axes. The growth range of *C. trifaia* is 5-25 cm high and 4-12 mm across. The ramuli are in three distinct longitudinal rows (two when juvenile) 3-5 mm long whilst the stolon bears scattered short spines about 0.5 mm long (Figure 3.1).[1]

Figure 3.1 *C. trifaria*, Spring Beach, Tasmania[1]

C. trifaria is usually found in sheltered, calm waters between depths of 2-31 m and is epilithic, often being found close to jetty piles or attached to sandy mud substrates. This green seaweed is a dominant plant on sand in sheltered bays in southern Tasmania

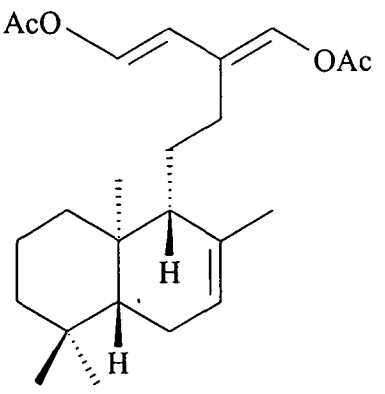
and sometimes forms a habitat with fish and an invertebrate community. During some of our collections two holothurian species were observed attached to the fronds of *C. trifaria.*

Previous studies by the Blackman research group in 1978 into a hexane extract of *C. trifaria* from Tinderbox, resulted in the isolation of trifarin **(1)** in 0.3% dry algal yield as an oil by silica gel chromatography. This was the first report of a 1,4-diacetoxybutadiene functionality in a natural product.[2]



(1)

An investigation into a collection of *C. trifaria* from Point Peron in Western Australia by Capon, Ghisalberti and Jefferies in 1983 isolated a bicyclic diterpene **(2)** in 0.4% dry algal yield. The diterpene **(2)** also possessed a 1,4-diacetoxybutadiene moiety.[3]

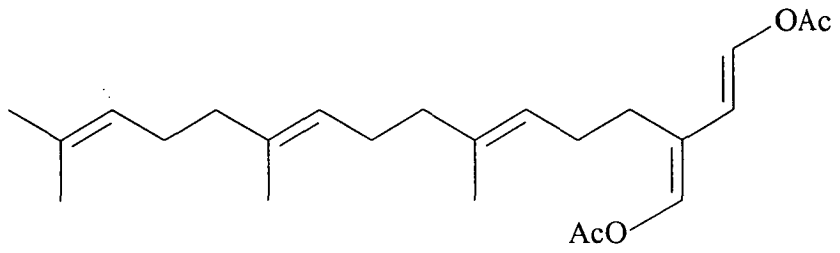


(2)

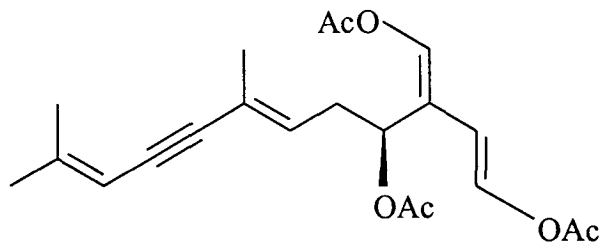
The diterpene **(2)** was isolated from a dichloromethane extract of the seaweed. The extract was fractionated through silicic acid using a solvent gradient and **(2)** eluted

with light petrol-dichloromethane (1:3) as a white crystalline compound. No trifarin **(1)** was isolated from the Point Peron collection.[3]

This chapter will detail studies of *C. trifaria* resulting in the isolation and structural elucidation of four novel monocyclic diterpenes containing an acetoxy or aldehyde functionality **(5)**, **(8)**, **(11)** and **(12)** and also trifarin **(1)**, the bicyclic diterpene **(2)**, didehydrotrifarin **(3)** and caulerpenyne **(4)**; all previously isolated from green algal species. Geographic variation and the instability of some secondary metabolites will also be discussed.



(3)



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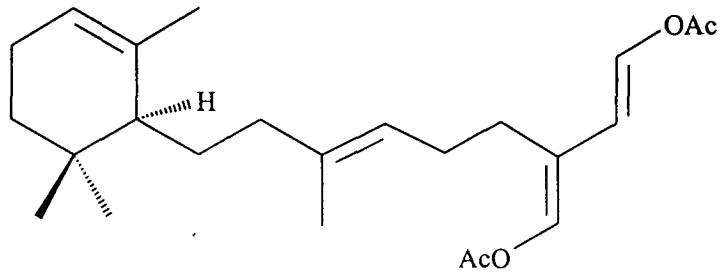

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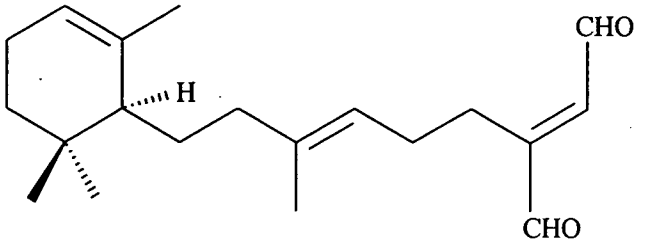

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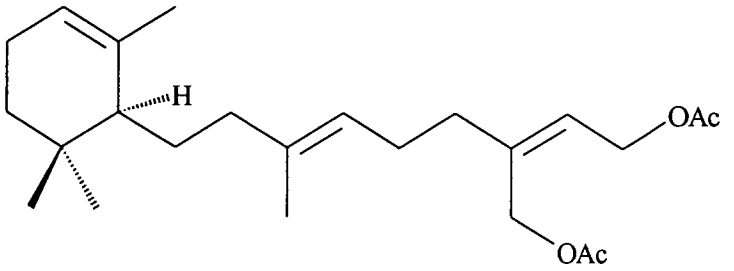
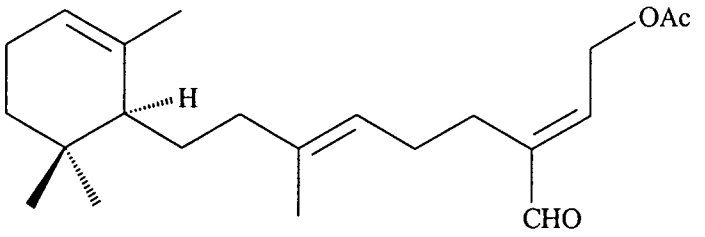


(11)



(12)

3.2 Collection, extraction and isolation

C. trifaria initially collected from Taroona, Tasmania was frozen within three hours of collection and stored at -20^{0}C until freeze drying. The DCM extract of the dried material was then subjected to ^{1}H n.m.r., t.l.c. and g.c.-m.s. analysis. The ^{1}H n.m.r. spectrum of the extract (Figure 3.2) showed signals in the 5.8-10.3 ppm region, characteristic of aldehyde and acetoxybutadiene moieties.

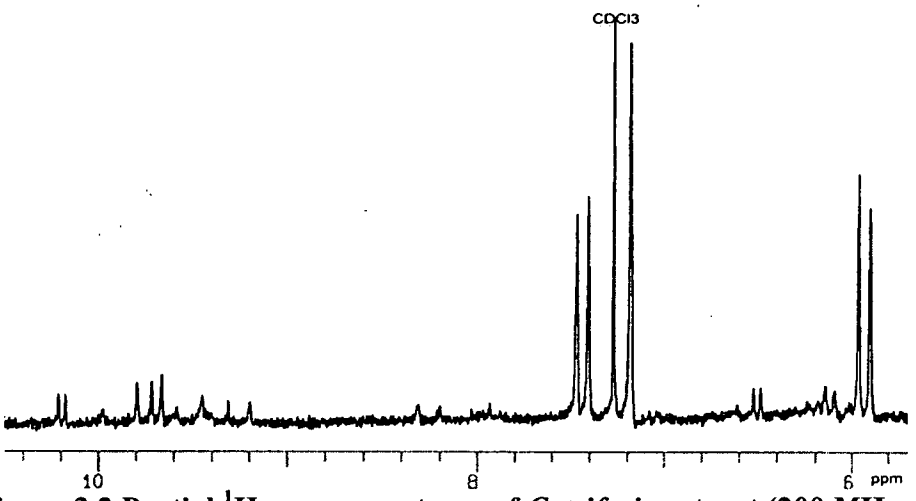


Figure 3.2 Partial ^{1}H n.m.r. spectrum of *C. trifaria* extract (200 MHz, $CDCl_3$)

The t.l.c. of the DCM extract in 1:4 ethyl acetate : DCM (Figure 3.3) revealed a number of UV active spots, indicating the likely presence of secondary metabolites.

Figure 3.3 T.l.c. of *C. trifaria* extract (1:4EtOAc:DCM) (vanillin spray)

The g.c.-m.s. profile of the *C. trifaria* DCM extract (Figure 3.4) indicated that compounds of a terpenoid nature were present in the molecular weight range below 430 a.m.u.

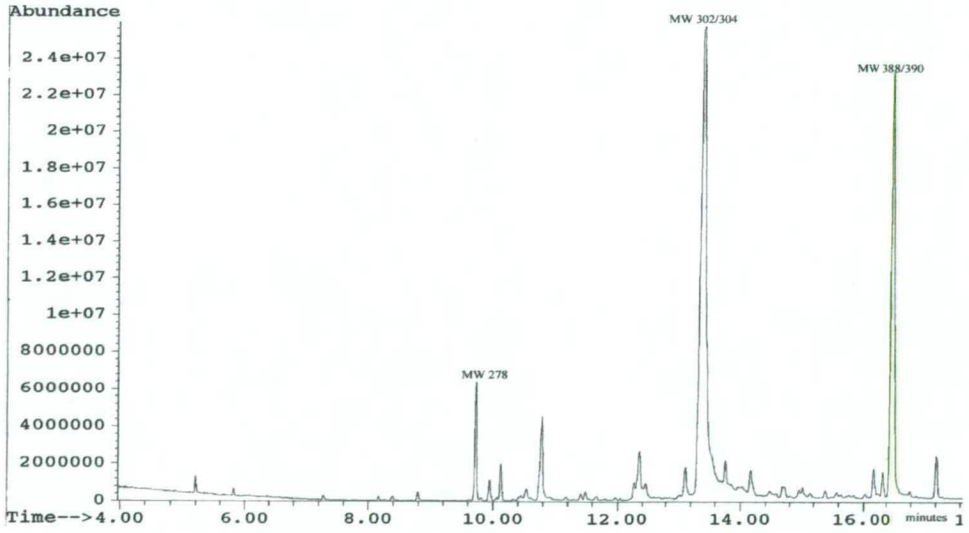
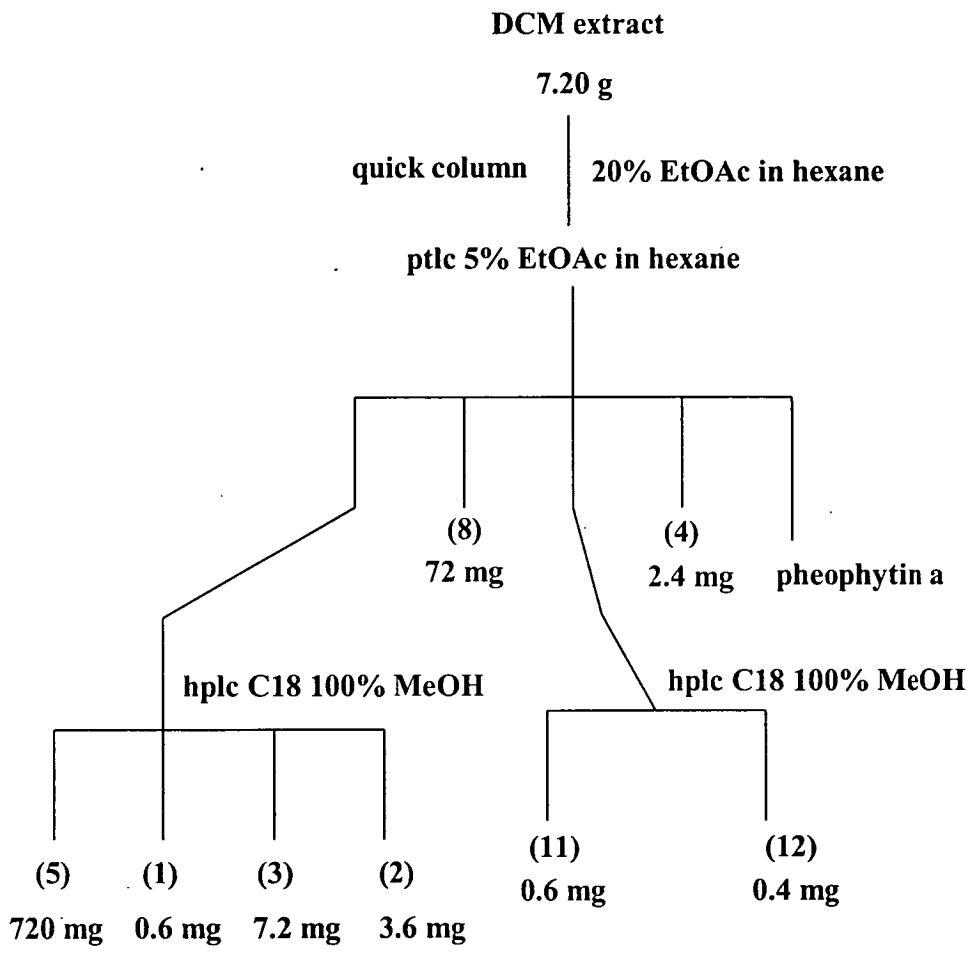


Figure 3.4 G.c-m.s. profile of *C. trifaria* extract (minutes)

The DCM extract of *C. trifaria* was then subjected to column and preparative thin layer chromatography as shown in Scheme 3.1.



Scheme 3.1 Purification scheme for DCM extract of *C. trifaria*

Extensive experimentation with flash chromatography, m.p.l.c and p.t.l.c resulted in optimum separation being achieved with p.t.l.c. in 5% ethyl acetate in hexane (Figure 3.5).

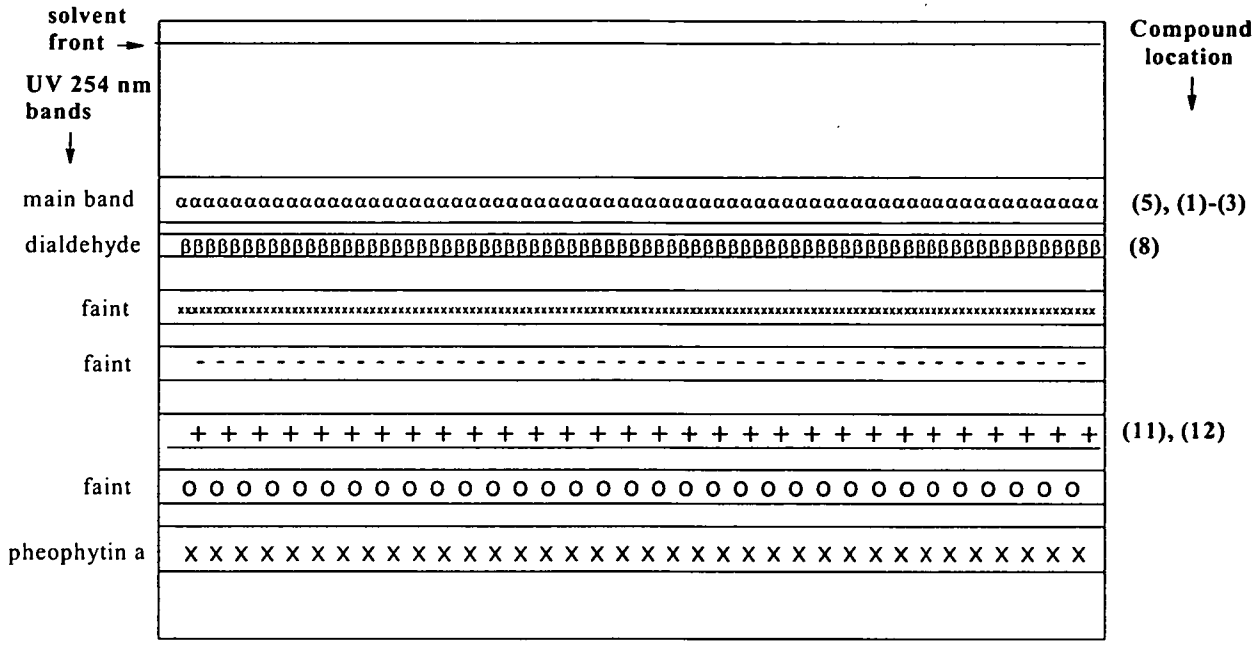


Figure 3.5 P.t.l.c. separation of *C. trifaria* extract in 5% EtOAc in hexane

After p.t.l.c. the main (least polar) fraction of the DCM extract was then subjected to reverse phase high performance liquid chromatography (C18, 100% methanol) to yield the novel acetoxybutadiene compound **(5)** as the major secondary metabolite and the known compounds **(1)-(3)**.

3.3 Structure Elucidation of (5)

The acetoxybutadiene compound **(5)** was isolated as a pale yellow oil $[\alpha_D] = -75^0$ (c=0.012M, EtOH) in 0.6% dry algal yield. By high resolution c.i. mass spectrometry, **(5)** was found to have a molecular formula of $C_{24}H_{36}O_4$ (M+1 389.26876, M+1calc. 389.26919). The u.v. spectrum (λ_{max} =251 nm, ε=22500, EtOH) and the i.r. spectrum (1208, 1370, 1629, 1756 cm^{-1}) were similar to those of trifarin **(1)**. However trifarin was found to have a molecular weight of 390, corresponding to a molecular formula of $C_{24}H_{38}O_4$ thus possessing an additional two hydrogen atoms. The e.i. mass spectrum of **(5)** differed substantially from that of trifarin **(1)**, suggesting that the two metabolites possessed structural differences.

The ^1H n.m.r. spectrum of **(5)** in $CDCl_3$ (Figure 3.6) contained signals characteristic of a 1,4-acetoxybutadiene moiety; two downfield protons at 7.44 and 7.15 ppm; the former a doublet coupled to a doublet at 5.92 ppm; The ^1H n.m.r. spectrum also contained six methyl signals at 2.16, 2.15, 1.66, 1.60, 0.91 and 0.86 ppm and methylene signals in the saturated aliphatic region between 1.0 and 2.4 ppm. Two further signals at 5.25 and 5.15 ppm indicated protons attached to carbon-carbon double bonds. The ^1H n.m.r. spectrum of **(5)** as such was almost identical to that of trifarin **(1)** differing only in the region 0.86 to 0.91 ppm. In **(5)** these signals were two singlets 11Hz apart (each 3 H) whilst in **(1)** the signal for the corresponding two methyl groups at 0.88 ppm (H 16, H 20) was a doublet (6 H, d *J* 7 Hz).[2]

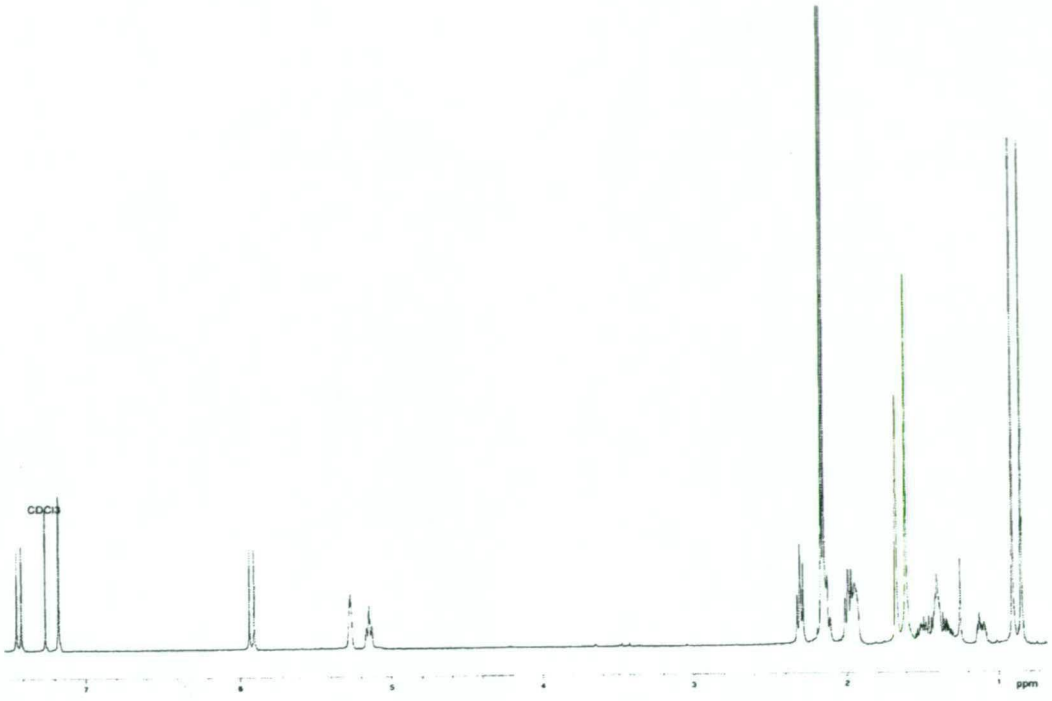


Figure 3.6 ^{1}H n.m.r spectrum of (5) (400 MHz, $CDCl_3$)

The ^{13}C n.m.r. spectrum of **(5)** (Figure 3.7) was almost identical to that of trifarin **(1)** with both spectra containing twenty four resonances; two carbonyl carbons, eight signals in the vinyl region and fourteen saturated aliphatic signals. In every case except one the signals were either identical or within 0.1 ppm (Table 3.1).

The multiplicities of the protonated carbons of **(5)** were determined by a distortionless enhancement through polarisation transfer (DEPT) experiment which revealed that the signal at 32.6 ppm in the ^{13}C spectrum of **(5)** corresponded to a quaternary C; in trifarin **(1)** the signal at 32.6 ppm corresponds to a methylene C. All other multiplicities of the ^{13} C signals for **(5)** and **(1)** were identical (Table 3.1).

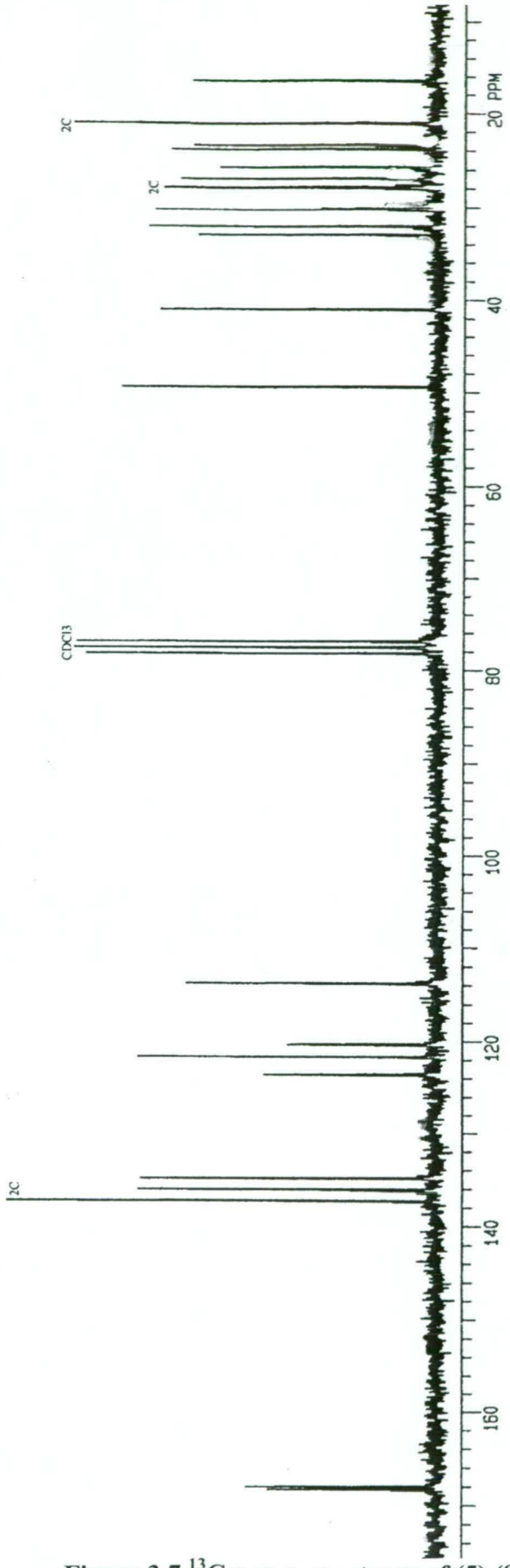


Figure 3.7 ^{13}C n.m.r. spectrum of (5) (90 MHz, $CDCl_3$)

Carbon	(1)	(5)	(8)	(11)	(12)	(6)	(7)
1	135.6 (d)	135.6 (d)	191.8 (d)	60.6 (t)	60.9 (t)	135.9 (d)	60.6 (t)
2	113.3 (d)	113.3 (d)	141.7 (d)	122.4 (d)	146.2 (d)	113.3 (d)	145.4 (d)
3	121.2 (s)	121.2 (s)	154.6 (s)	139.7 (s)	144.5 (s)	122.4 (s)	146.1 (s)
4	25.4 (t)	25.3 (t)	24.4 (t)	25.8 (t)	25.0 (t)	26.1 (t)	23.1 (t)
5	26.6 (t)	26.6 (t)	27.9 (t)	27.0 (t)	26.9 (t)	29.4 (t)	25.1 (t)
6	123.0 (d)	123.1 (d)	121.6 (d)	122.4 (d)	122.5 (d)	49.3 (d)	49.6 (d)
7	136.7 (s)	136.8 (s)	139.9 (s)	136.9 (s)	137.6 (s)	136.3 (s)	136.0 (s)
8	40.6 (t)	40.6 (t)	40.9 (t)	40.7 (t)	40.7 (t)	120.4 (d)	120.8 (d)
9	29.9 (t)	29.8 (t)	30.1 (t)	29.9 (t)	29.9 (t)	23.1 (t)	29.9 (t)
10	119.8 (d)	49.0 (d)	49.5 (d)	49.1 (d)	49.2 (d)	31.8 (t)	31.8 (t)
11	136.7 (s)	136.8 (s)	137.0 (s)	137.3 (s)	136.8 (s)	32.6 (s)	32.6 (s)
12	32.6 (t)	119.9 (d)	120.5 (d)	120.0 (d)	120.0 (d)	134.2 (d)	194.2 (d)
13	23.0 (t)	23.1 (t)	23.5 (t)	23.2 (t)	23.3 (t)	23.2 (q)	23.4 (q)
14	31.7 (t)	31.6 (t)	32.1 (t)	31.7 (t)	31.8 (t)	27.3 (q)	27.5 (q)
15	49.0 (d)	32.6 (s)	33.0 (s)	32.7 (s)	32.8 (s)	27.6 (q)	27.7 (q)
16	135.4 (d)	134.4 (d)	195.5 (d)	67.2 (s)	194.0 (d)		
17	16.1 (q)	16.1 (q)	16.7 (q)	16.2 (q)	16.3 (q)		
18	23.4 (q)	23.5 (q)	23.9 (q)	23.6 (q)	23.7 (q)		
19	27.5 (q)	27.4 (q)	27.9 (q)	27.6 (q)	27.7 (q)		
20	27.5 (q)	27.6 (q)	27.7 (q)	27.8 (q)			
$O\underline{C}OCH_3$	2x 167.3 (s)	2x 167.4 (s)		171.1 (s) 170.8 (s)	171.0 (s)	2x 167.5 (s)	
$OCO\underline{C}H_3$	2x 20.6 (q)	2x 20.7 (q)		2x 21.1 (q)	21.0 (q)	2x 20.6 (q)	

[A] spectra obtained in $CDCl_3$ at 100 MHz; [B] multiplicities assigned from DEPT spectra

Table 3.1 ^{13}C n.m.r. data (ppm) for compounds (1), (5), (8), (11), (12)[A,B] (6) and (7)

A heteronuclear correlation (HETCOR) n.m.r. experiment (Figure 3.8) provided information on the ^{13}C-^{1}H one bond connections in **(5)** indicating that there were two sets

of non-equivalent protons (H 9, H 14) and confirming that there were six quaternary carbon atoms present in the structure of **(5)** (Table 3.2).

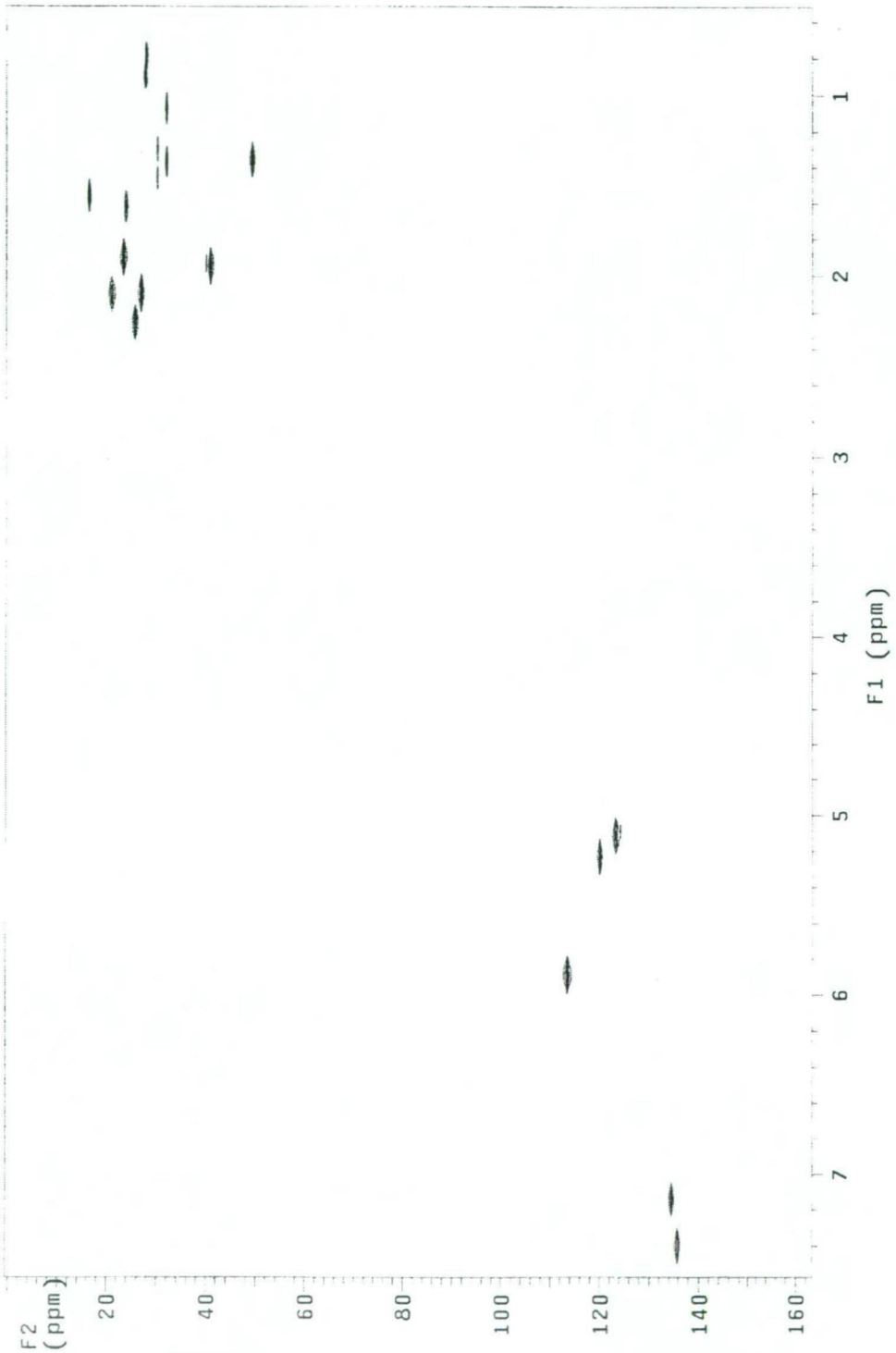


Figure 3.8 HETCOR n.m.r. spectrum of (5) (100 MHz, $CDCl_3$)

A correlation through multiple-quantum coherence (INADEQUATE) n.m.r. experiment (Figure 3.9) was then carried out to obtain information on the carbon-carbon connectivities in **(5)**.

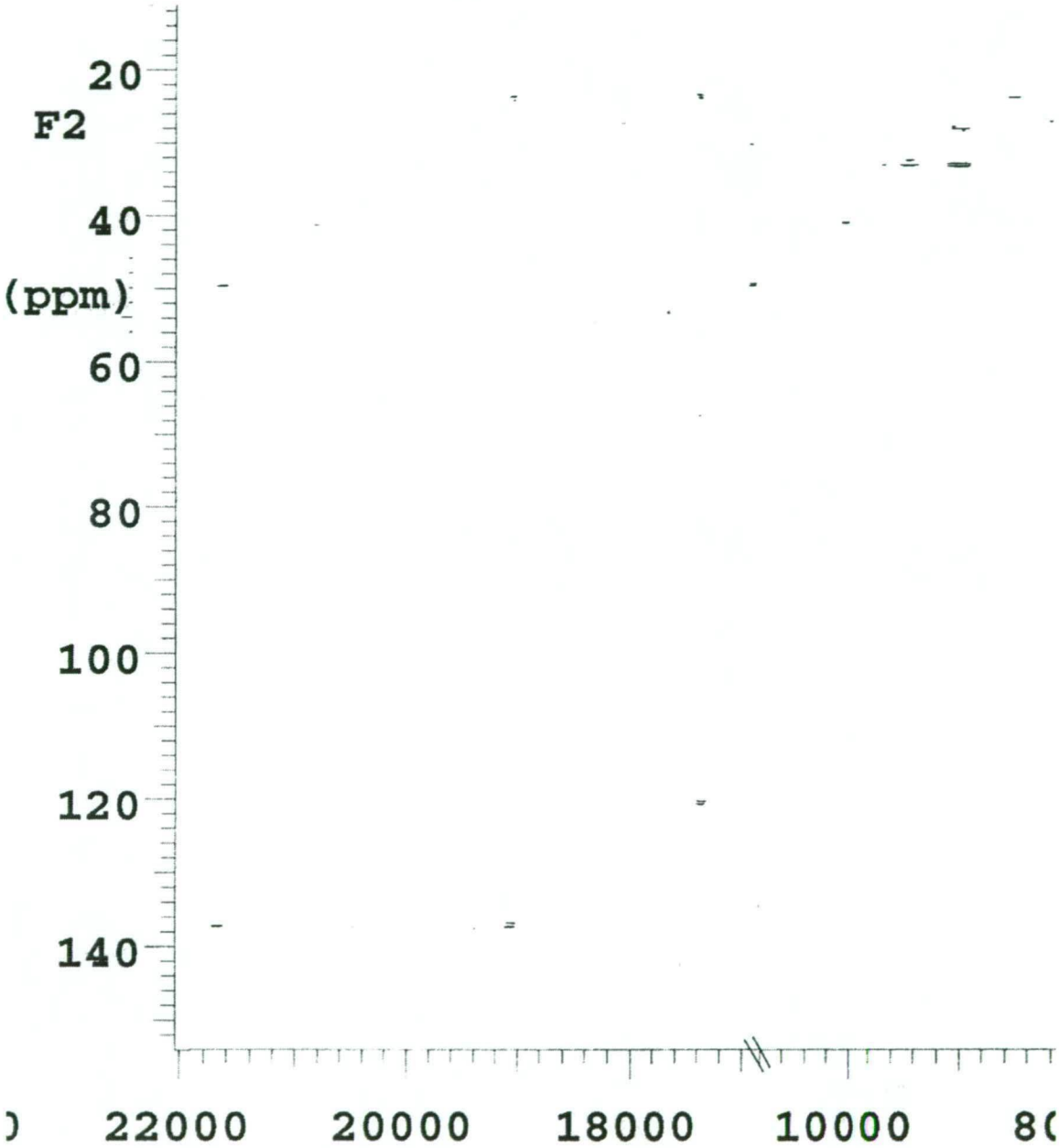
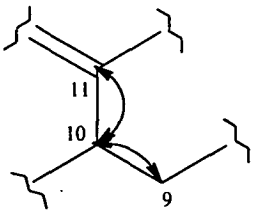
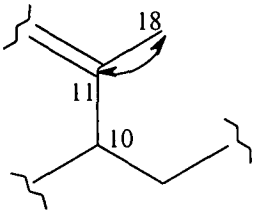


Figure 3.9 INADEQUATE spectrum of (5) ($CDCl_3$, 100 MHz)

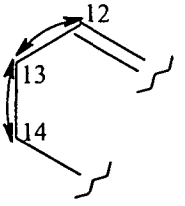
The methylene ^{13}C signal at 29.8 ppm (C 9) showed a connection to the methine ^{13}C signal at 49.0 ppm (C 10) which in turn showed a connection to the quaternary ^{13}C signal at 136.8 ppm (C 11).



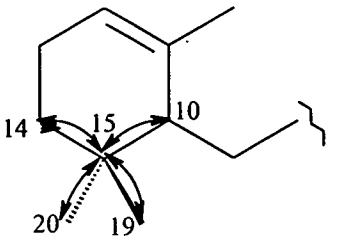
This C 11 quaternary signal also showed a connection to the methyl ^{13}C signal at 23.5 ppm (C 18).



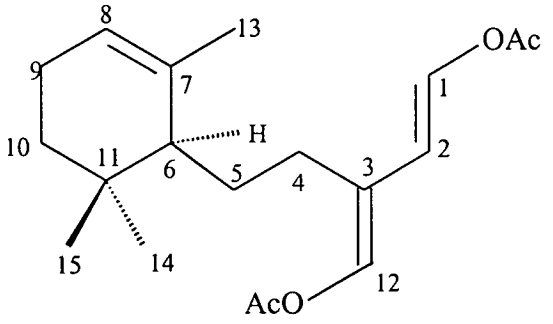
The methine ^{13}C signal at 119.9 ppm (C 12) showed a connection to the methylene ^{13}C signal at 23.1 ppm (C 13) which in turn showed a connection to the methylene ^{13}C signal at 31.6 ppm (C 14).



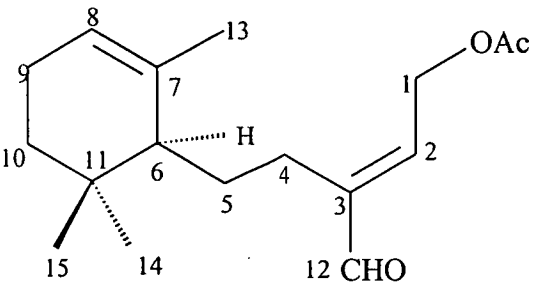
This C 14 methylene signal also showed a connection to the quaternary ^{13}C signal at 32.6 ppm (C 15) which in turn showed a connection to the methine ^{13}C signal at 49.0 ppm (C 10) and the ^{13}C signals attributed to the geminal dimethyl groups at 27.4 and 27.6 ppm (C 19/20).



Thus the results of the INADEQUATE n.m.r. experiment suggested that a 1,5,5-trimethylcyclohexene moiety was present in the structure of **(5)**. Comparison of ^{13}C n.m.r. data for **(5)** with those of **(6)** and **(7)** (Table 3.1), previously isolated from *C. flexilis var. muelleri*[4] further supported the inclusion of a 1,5,5-trimethylcyclohexene moiety in the structure of **(5)**.



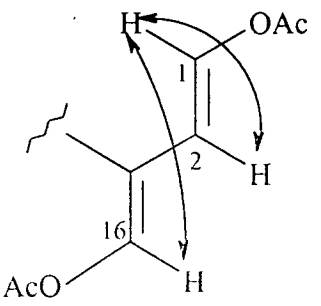
(6)



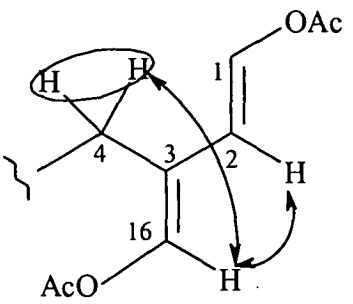
(7)

Correlation spectroscopy (COSY) n.m.r. experiments, both short and long range, (Figures 3.10- 3.12) were then carried out on **(5)** to ascertain the ^{1}H-^{1}H couplings within the molecule (Table 3.2):

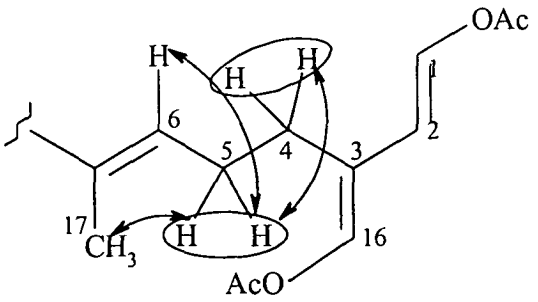
H 1 at 7.44 ppm showed coupling to H 2 at 5.92 ppm and also long range coupling (^{5}J) to H 16 at 7.15 ppm.



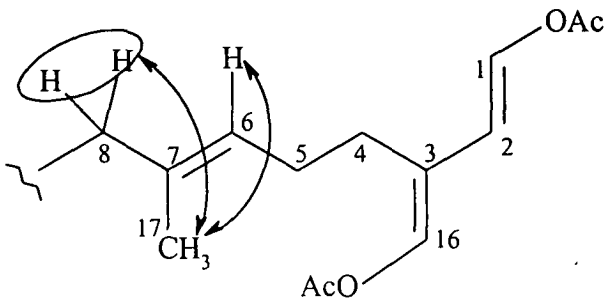
H 16 in turn showed long range coupling (^4J) to H 2 at 5.92 ppm and to the H 4 protons at 2.30 ppm.



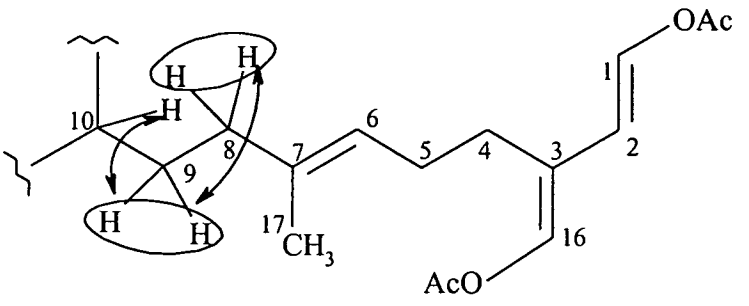
The H 4 protons also showed coupling to the H 5 protons at 2.13 ppm which in turn showed coupling to the H 6 methine proton at 5.15 ppm and long range coupling (^5J) to the H 17 methyl protons at 1.60 ppm.



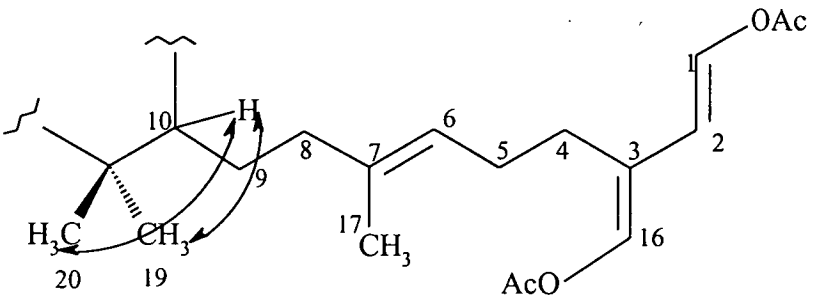
H 6 also showed long range coupling (^4J) to the H 17 methyl protons at 1.60 ppm. Furthermore these H 17 protons showed long range coupling (^4J) to the H 8 protons at 1.98 ppm.



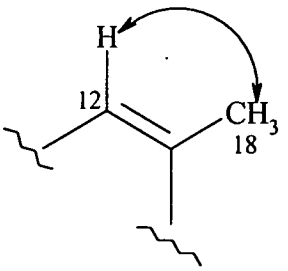
The H 8 protons in turn showed coupling to the H 9 protons at 1.33 and 1.50 ppm. The COSY spectrum showed overlap in the region 1.30 to 1.50 ppm which was however consistent with the H 9 protons coupling to the H 10 methine proton at 1.40 ppm.



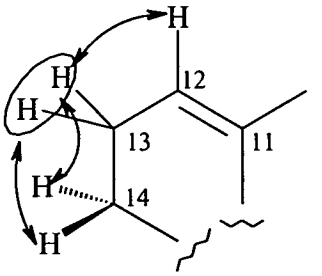
H 10 also showed long range (^4J) coupling to the geminal dimethyl groups at 0.91 ppm and 0.86 ppm (H 19, H 20).



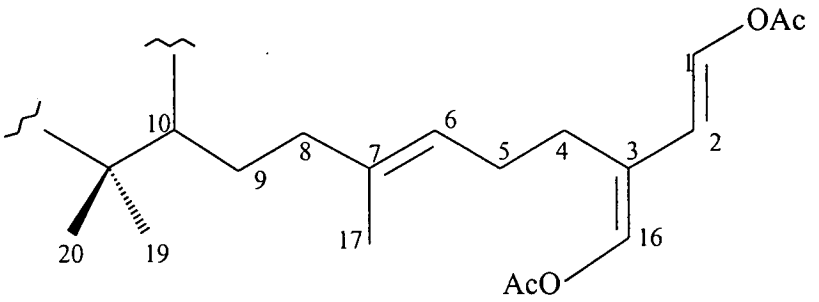
The H 18 methyl group protons at 1.66 ppm showed long range coupling (^4J) to the H 12 methine proton at 5.25 ppm.

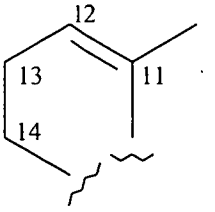


H 12 in turn showed coupling to the H 13 protons at 1.95 ppm which in turn showed coupling to the H 14 protons at 1.41 and 1.10 ppm.

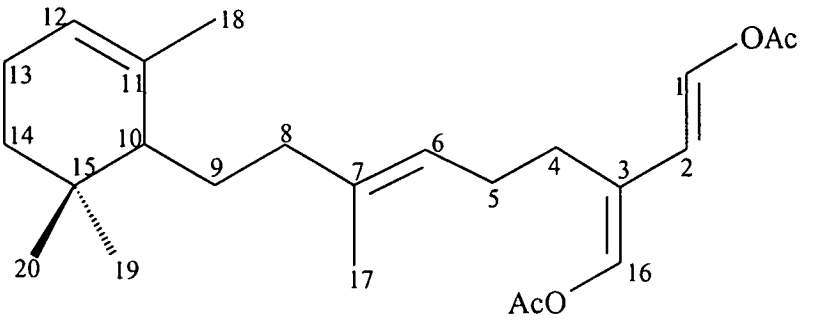


Thus the results of the COSY experiments supported the inclusion of the following substructures in the structure of **(5)**:





Together with the results of the INADEQUATE experiment, these substructures could be linked to give the following structure for **(5)**:



(5)

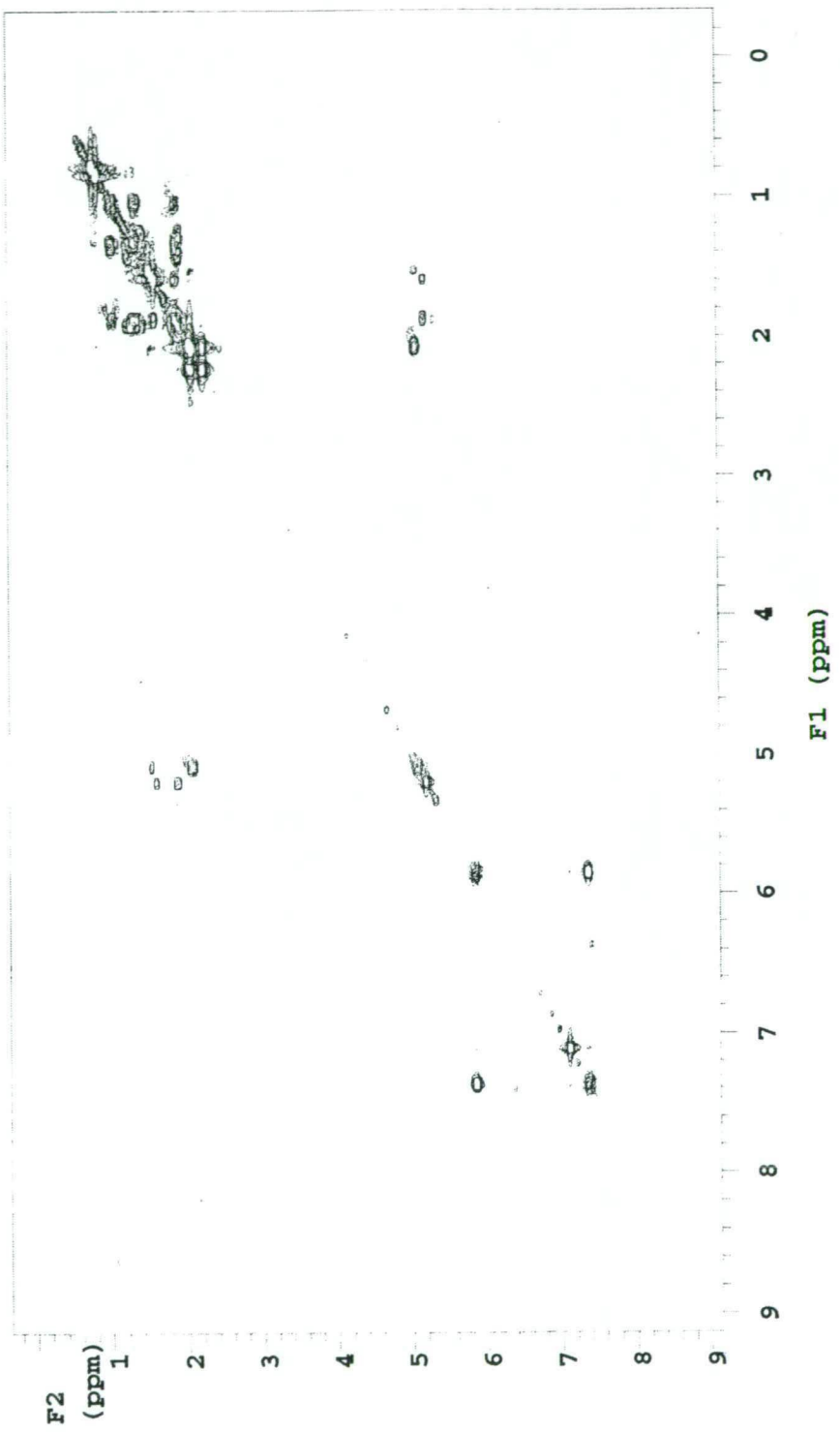


Figure 3.10 COSY spectrum of (5) (400MHz, $CDCl_3$)

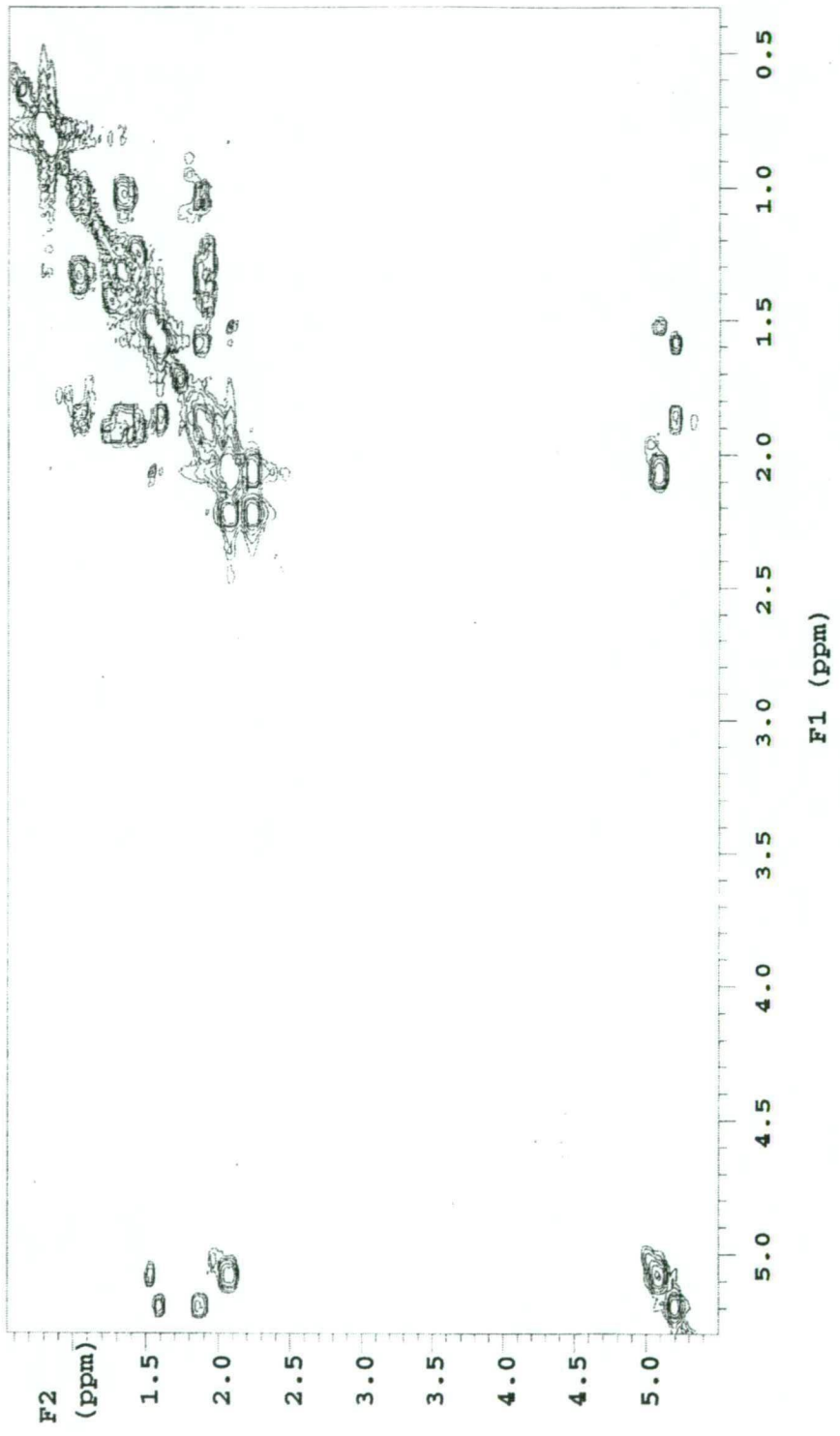


Figure 3.11 COSY spectrum of (5); enlargement of spectrum 3.10

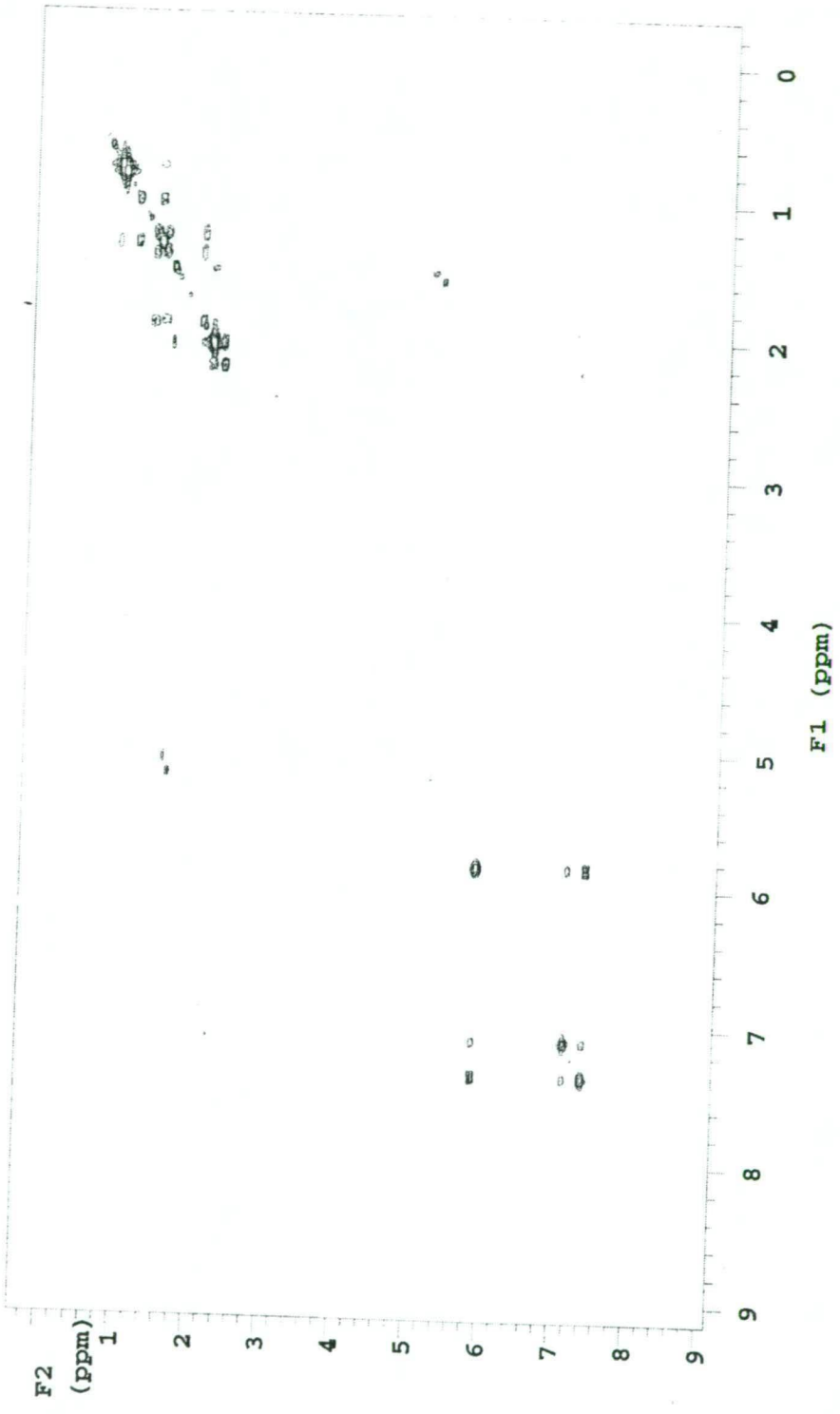
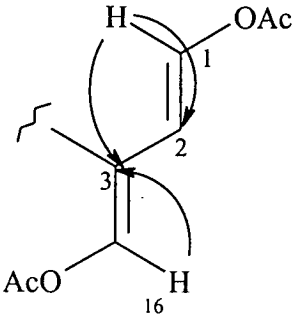


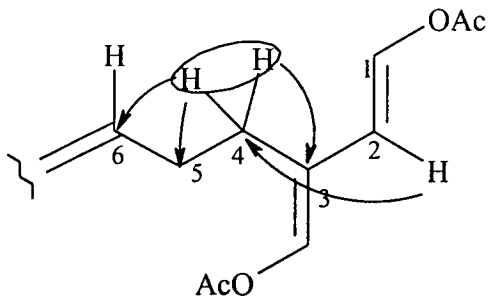
Figure 3.12 COSY n.m.r. spectrum of (5); long range (400MHz, $CDCl_3$)

The complete structure of **(5)** was confirmed by a heteronuclear multiple bond correlation (HMBC) n.m.r. experiment which provided information on the H-C-C and H-C-C-C connectivities within **(5)**:

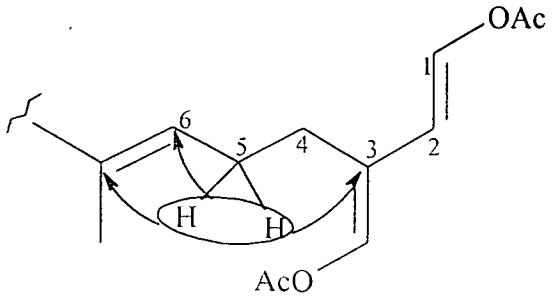
The H 1 proton at 7.44 ppm showed a correlation to the C 2 signal at 113.3 ppm and the C 3 signal at 121.2 ppm whilst the H 16 proton at 7.15 ppm showed a correlation to the C 3 signal at 121.2 ppm.



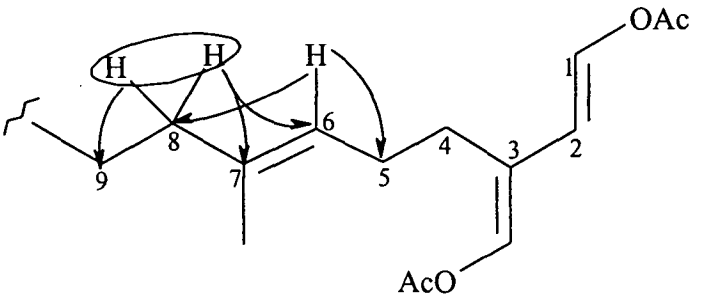
The H 2 proton at 5.92 ppm showed a correlation to the C 4 signal at 25.3 ppm. The H 4 protons at 2.30 ppm were correlated to the C 3 signal at 121.2 ppm, the C 5 signal at 26.6 ppm and the C 6 signal at 123.1 ppm.



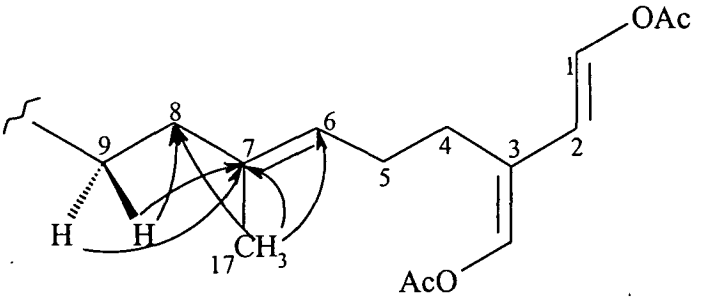
The H 5 protons at 2.13 ppm showed correlations to the C 3 signal at 121.2 ppm, the C 6 signal at 123.1 ppm and the C 7 signal at 136.8 ppm.



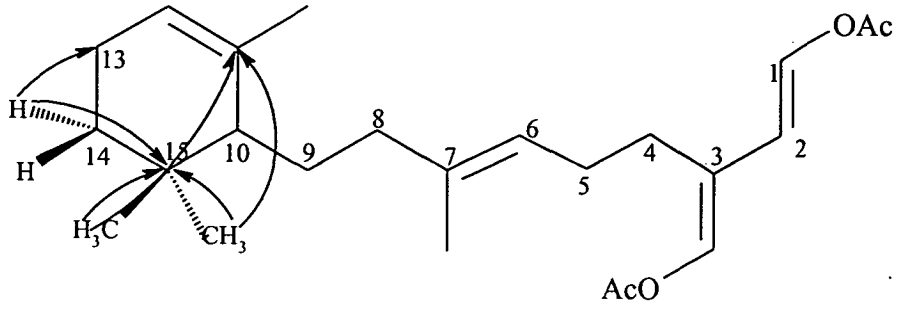
The H 6 proton at 5.15 ppm showed correlations to the C 5 signal at 26.6 ppm and the C 8 signal at 40.6 ppm. The H 8 protons at 1.98 ppm showed correlations to the C 6 signal at 123.1 ppm, the C 7 signal at 136.8 ppm and the C 9 signal at 29.8 ppm.



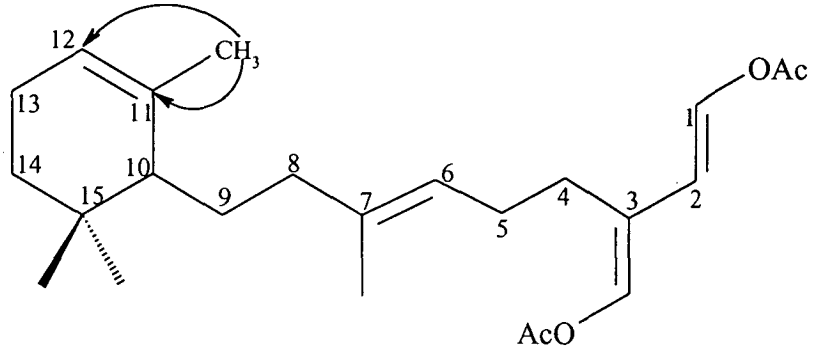
The H 9 proton resonating at 1.50 ppm showed a correlation to the C 7 signal at 136.8 ppm whilst the H 9 proton resonating at 1.33 ppm showed correlations to the C 7 signal and also to the C 8 signal at 40.6 ppm whilst the H 17 methyl protons at 1.60 ppm showed correlations to the C 6 signal at 123.1 ppm, the C 7 signal at 136.8 ppm and the C 8 signal at 40.6 ppm



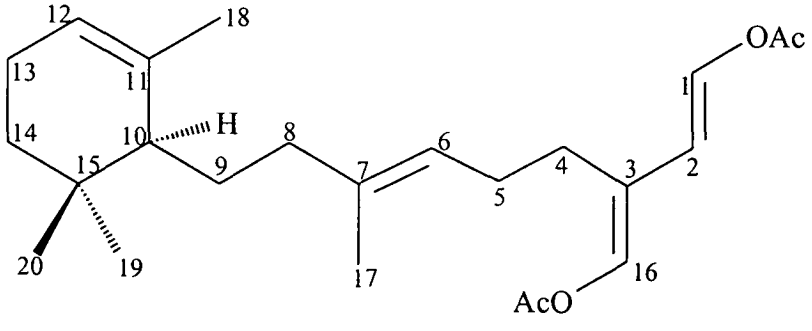
The H 14 proton at 1.10 ppm showed a correlation to the C 15 signal at 32.6 ppm and the C 13 signal at 23.1 ppm whilst the geminal dimethyl protons (H 19 and H 20) at 0.91 ppm and C 20 at 0.86 ppm respectively showed correlations to the C 15 signal at 32.6 ppm and the C 11 signal at 136.8 ppm.



Finally the H 18 methyl protons at 1.66 ppm showed correlations to the C 11 signal at 136.8 ppm and the C 12 signal at 119.9 ppm.



Thus the HMBC correlations confirmed the following structure for **(5)**:



(5)

The absolute stereochemistry of the chiral centre at C 10 was assigned as *S* based on the negative sign for the optical rotation of **(5)**. The reasons for this assignment will be discussed later in this chapter.

[Note: The HMBC spectrum for **(5)** was not able to be located at the time of thesis writing, however was documented at the time of publication of the novel *C. trifaria* metabolites in the *Aust. J. Chem.* article in 2000. Another spectrum could not be run due to the delay between this work in 1998 and thesis writing in 2002 and the remote location of the candidate.]

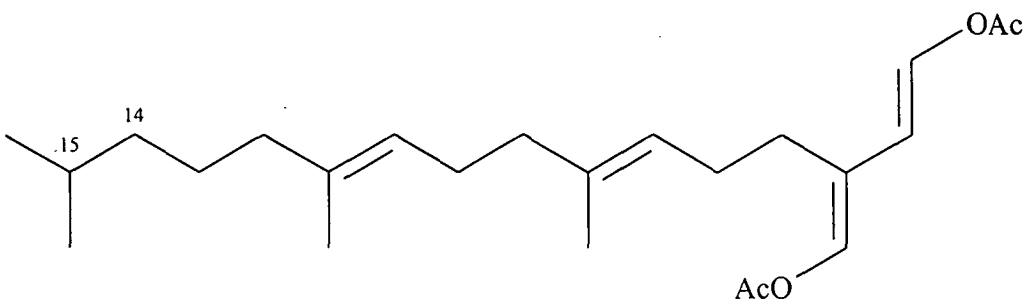
The *E*- geometry of the C 1-C 2 olefin in the diacetoxybutadiene system followed from the value of its coupling constant (*J* 13 Hz) between the vicinal protons; however that of the trisubsitituted olefin (C 3) has not been determined unambiguously. In a number of naturally occurring 1,4-diacetoxy-1,3-butadienes[5,2,6] the *E,E*-configuration has been assumed because of the close agreement with the spectral data of model *E,E*- and *Z,Z*-compounds.[7] In the case of caulerpenyne **(4)** the n.O.e results suggested an *E*-configuration for the trisubstituted double bond. Since the spectral properties of **(5)** are consistent with those of **(4)**, an *E,E*- configuration seems probable. The stereochemistry of the C 6-C 7 olefin was determined to be *E* by comparison of the shielded ^{13}C value for the vinyl methyl (16.7 ppm) with that of similar compounds **(1)** (Table 3.1) and **(3)**.[6]

Carbon	^{13}C	^{1}H	COSY	HMBC	INADEQUATE
1	135.6 d	7.44 d *J* 12.5 Hz	H 2	C 2, C 3	
2	113.3 d	5.92 d *J* 12.5 Hz	H 1, H 4	C 4	
3	121.2 s	----	----		
4	25.3 t	2.30 t *J* 8.0 Hz	H 5	C 3, C 5, C 6	
5	26.6 t	2.13 m	H 4, H 6	C 3, C 6, C 7	
6	123.1 d	5.15 br t *J* 8.0 Hz			
7	136.8 s	----			
8	40.6 t	1.98 m	H 9	C 6, C 7, C 9	
9	29.8 t	1.50/1.33 m	H 8, H 10	C 7	
10	49.0 d	1.40 m	H 9, H 19		C 11, C 9
11	136.8 s	----			
12	119.9 d	5.25 br s	H 13, H 18		
13	23.1 t	1.95 m	H 12, H 14		C 12
14	31.6 t	1.41 m	H 14		
15	32.6 s	----			C 14, C 10, C 19
16	134.4 s	7.15 s		C 3	
17	16.1 q	1.60 q	H 6	C 6, C 7, C 8	
18	23.5 q	1.66 s	H 12, H 13	C 11, C 12	C 11
19	27.4 q	0.91 s	H 10	C 11, C 15	
20	27.6 q	0.86 s	H 10	C 11, C 15	

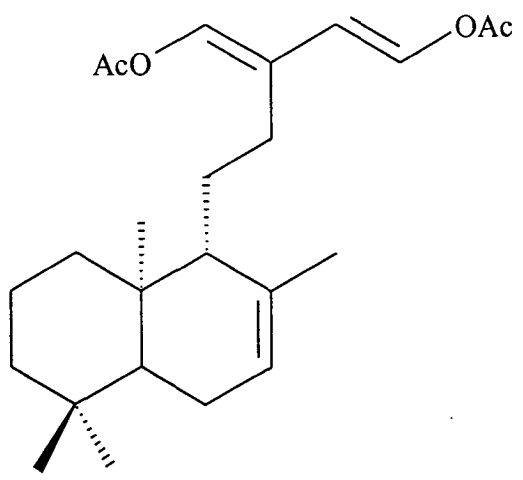
Table 3.2 HETCOR, COSY, HMBC and INADEQUATE data for (5) ($CDCl_3$)

3.4 Isolation of compounds (1), (2) and (3)

The reverse phase h.p.l.c. (C18, 100% MeOH) which yielded **(5)** as the major secondary metabolite (0.6% dry algal yield) also yielded a further three secondary metabolites in 0.0005%, 0.003% and 0.006% dry algal yields respectively. Although these three metabolites were present in minute quantities compared to the major metabolite **(5)** (<1%) it was thought useful to identify them in view of the different metabolites **(2)**[3] and **(1)**[2] previously isolated from *C. trifaria*



(1)



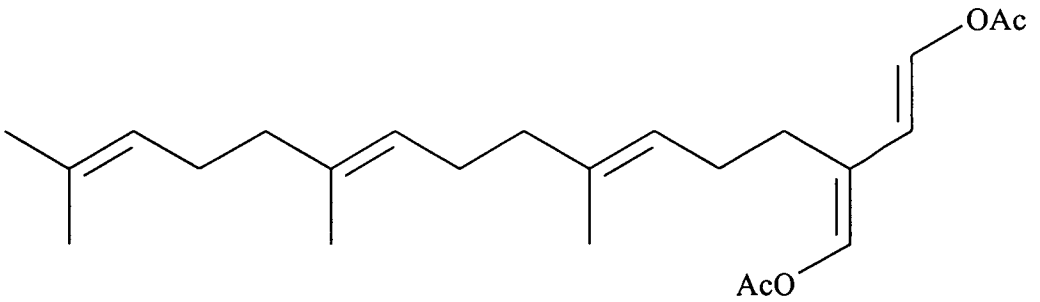
(2)

The presence of the bicyclic compound **(2)** in this collection of *C. trifaria* was suggested by the presence of small peaks in the ^{13}C n.m.r. spectrum of the p.t.l.c. fraction, which matched the ^{13}C n.m.r. literature values given for compound **(2)**.[3]

Subsequent analysis of the n.m.r. spectral data and m.s. data (Appendix 1, Figures 1.7-1.9) of the metabolite present in 0.003% dry algal yield after h.p.l.c., confirmed that this compound was identical to **(2)**.[3]

The metabolite **(3)** present in 0.006% dry algal yield possessed ^{1}H n.m.r. spectral data almost identical to trifarin **(1)**[2] (Appendix 1, Figure 1.5); indeed the e.i. mass spectra of this metabolite was identical to that of trifarin **(1)** (Appendix 1, Figure 1.10). The ^{13}C n.m.r. spectrum of **(3)** (Appendix 1, Figure 1.3), however included signals at 131.4 ppm and 124.4 ppm not present in trifarin **(1)** whilst the ^{13}C signals corresponding to C 14 and C 15 in trifarin **(1)** were absent.

The DEPT n.m.r. spectral analysis (Appendix 1, Figure 1.4) of **(3)** indicated that an additional double bond was present at C 14 - C 15 in this metabolite. This was confirmed by COSY and HMBC n.m.r. experiments (Appendix 1, Figures 1.5, 1.6) which indicated that the structure of **(3)** was:



(3)

An examination of the literature revealed that this compound was didehyrotrifarin; first isolated in 1979 by Wells and Barrow from *Chlorodesmis fastigiata*; a green alga common on the reef flats of the Great Barrier Reef.[12] The ^{13}C n.m.r. spectral data contained in the literature gave values for 22 carbon atoms; however there are 24 carbon

atoms in the structure of **(3)**. The additional values obtained by our ^{13}C n.m.r. experiments are 135.7 ppm (=$\underline{C}$H) and 29.9 ppm (-$\underline{C}H_2$-). Further reference will be made to the n.m.r. spectral data of didehyrotrifarin in Chapter 4. The mass spectral data of **(3)** (Appendix 1, Figure 1.2) matched the literature values given by Wells and Barrow[12] for didehyrotrifarin: *m/z* 328 (M^+-60), 286, 191, 109, 69, 43. Didehyrotrifarin **(3)** more recently has been reported from another investigation into *C. fastigiata* in 1990, by Wright and Coll[10] from a collection from John Brewer Reef in North Queensland.

The metabolite isolated after h.p.l.c. in 0.0005% dry algal yield gave poor spectral data due to its scarcity, however its ^1H n.m.r spectrum included signals typical of a diacetoxybutadiene. The e.i. mass spectrum was identical to that of didehyrotrifarin **(3)** (Appendix 1, Figure 1.2) and it seems reasonable to assume on this basis that this metabolite is trifarin **(1)** as it has been reported in the literature that the mass spectra of the two metabolites **(1)** and **(3)** are indistinguishable[12] and trifarin **(1)** had been previously reported from *C. trifaria.*[2]

The initial investigation twenty four years ago by Blackman and Wells, into a Tinderbox collection of *C. trifaria* yielded **(1)** as the major secondary metabolite.[2] A number of collections of *C. trifaria* from various locations along the East Coast of Tasmania, including Tinderbox, were investigated during the course of this thesis. In all cases **(5)** was present in much higher concentrations than **(1)**, **(2)** or **(3)**. The Western Australian investigation into *C. trifaria* yielded **(2)** as the major secondary metabolite. It is possible that the chemical variation in the concentration of these secondary metabolites is due to environmental factors such as herbivory fluctuations in the areas where *C. trifaria* is found; for example Tinderbox was declared a marine reserve in 1981 three

years after the initial investigation. The presence of compounds **(1)**, **(2)** and **(3)** in our current investigation as minor secondary metabolites suggests a biosynthetic link between these and the other secondary metabolites of *C. trifaria*.

3.5 Structure elucidation of compound (8)

The next most polar compound **(8)** was isolated after p.t.l.c. as an unstable oil $[\alpha_D]=-71^0$ (c=0.007M, EtOH) in 0.06 % dry algal yield. By high resolution e.i. mass spectrometry, **(8)** was found to have a molecular formula of $C_{20}H_{30}O_2$ (*m/z* 302.22506, calc. 302.22458).

The ^1H n.m.r. spectrum of **(8)** in $CDCl_3$ (Figure 3.13) contained signals typical of aldehyde protons at 10.20 and 9.66 ppm; the former a doublet coupled to a doublet integrating to one proton at 6.51 ppm. Strong infrared absorption at 1685 cm^{-1} and u.v. absorption at 227 nm (ε = 6700, EtOH) indicated that **(8)** was an α,β-unsaturated dialdehyde. The remaining signals in the ^1H n.m.r. spectrum of **(8)** were two signals at 5.28 and 5.08 ppm corresponding to protons attached to carbon-carbon double bonds; methylene signals in the saturated aliphatic region between 1.0 and 2.8 ppm and four methyl signals at 1.60, 1.52, 0.90 and 0.85 ppm.

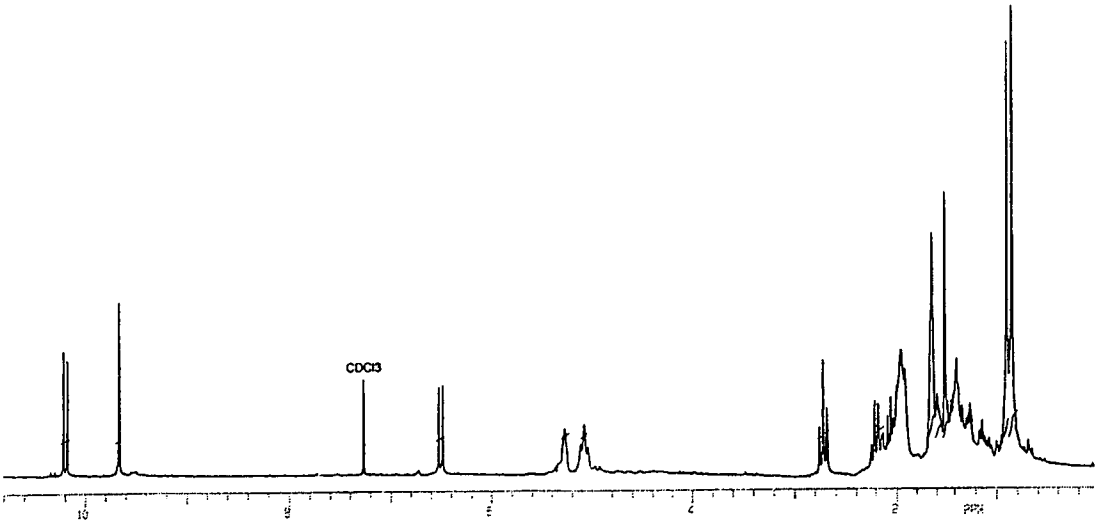


Figure 3.13 ^1H n.m.r. spectrum of (8) (200 MHz, $CDCl_3$)

The ^{13}C spectrum (Figure 3.14) of **(8)** contained twenty resonances; two signals at 191.8 and 195.5 ppm characteristic of aldehyde moieties, signals at 154.6 and 141.7 ppm typical of carbon-carbon double bonds attached to an aldehyde group; a four further signals in the vinyl region between 120 and 140 ppm and twelve signals in the saturated aliphatic region between 50 and 10 ppm (Table 3.1).

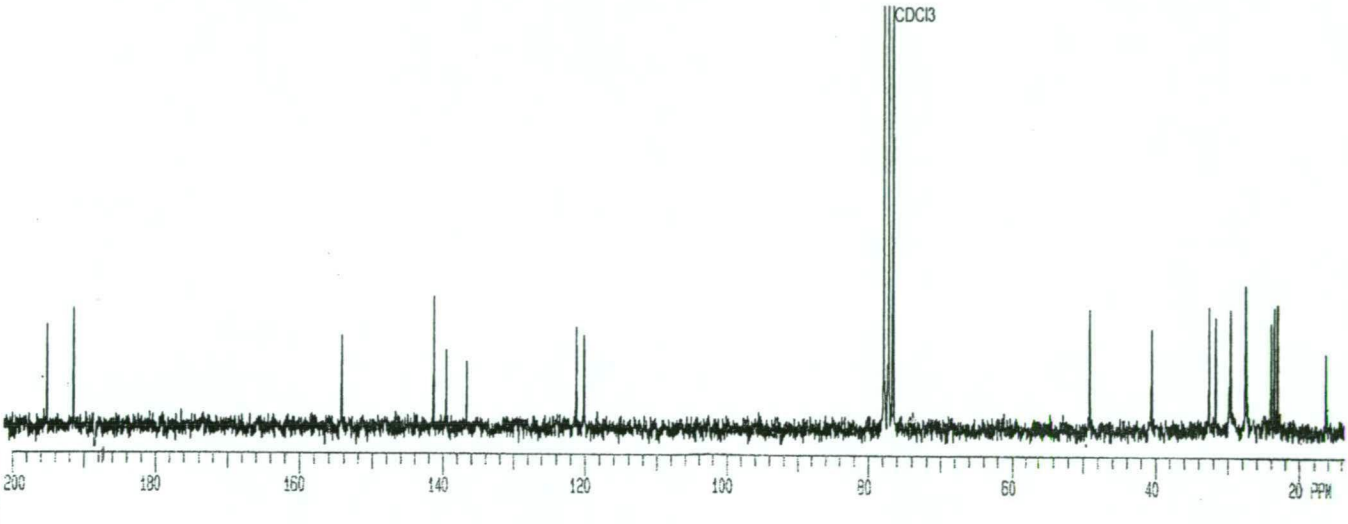


Figure 3.14 ^{13}C n.m.r. spectrum of (8) (90 MHz, $CDCl_3$)

The multiplicity of the protonated carbons of **(8)** was determined by a DEPT experiment (Figure 3.15) indicating that **(8)** possessed six methine, six methylene and four methyl groups. The four remaining ^{13}C resonances at 154.6, 139.9, 137.0 and 33.0 ppm were therefore attributed to quaternary carbon atoms (Table 3.1).

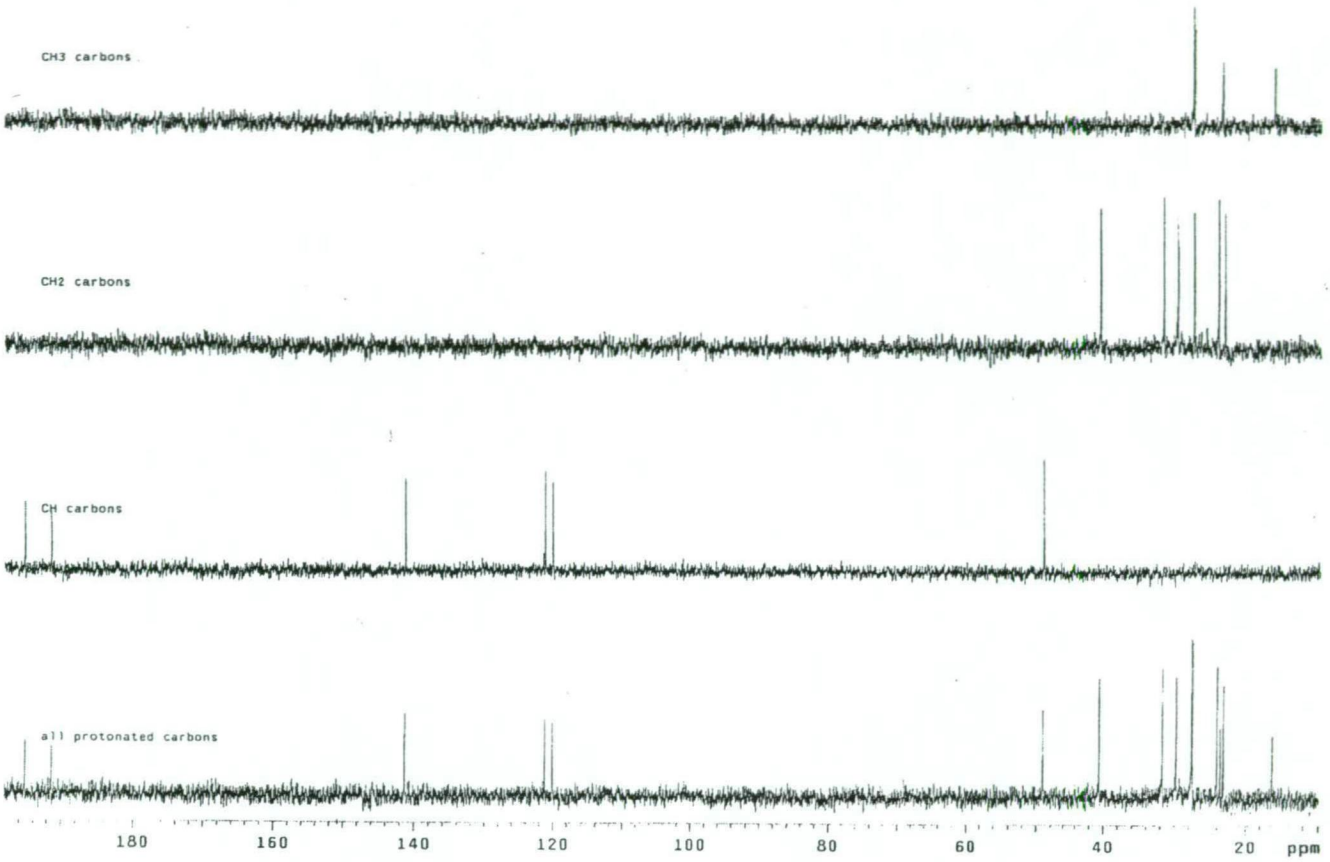
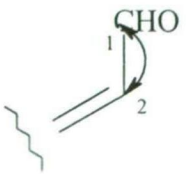


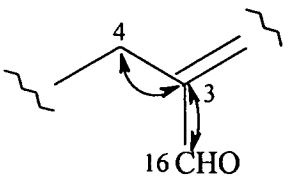
Figure 3.15 DEPT n.m.r. spectrum of (8) (100 MHz, $CDCl_3$)

A GHMQC experiment provided the ^{13}C–^{1}H connections in **(8)** (Table 3.3) which in conjunction with the DEPT analysis and an INADEQUATE experiment (Figures 3.16/17); provided the following information on the carbon-carbon connectivities in **(8)**:

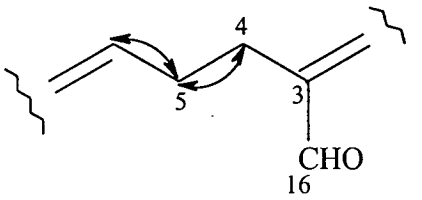
The aldehydic ^{13}C signal at 191.8 ppm (C 1) showed a correlation to the methine ^{13}C signal at 141.7 ppm (C 2).



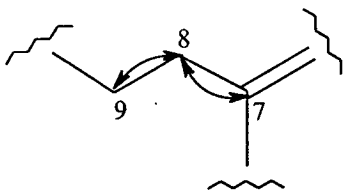
The quaternary ^{13}C signal at 154.6 ppm (C 3) showed correlations to the methylene ^{13}C signal at 24.4 ppm (C 4) and also to the aldehydic ^{13}C signal at 195.5 ppm (C 16).



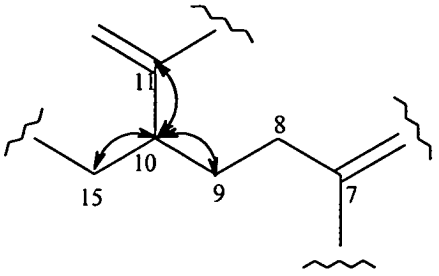
The ^{13}C signal at 24.4 ppm (C 4) also showed a correlation to the methylene ^{13}C signal at 27.9 ppm (C 5) which in turn showed a correlation to the methine ^{13}C signal at 121.6 ppm (C 6).



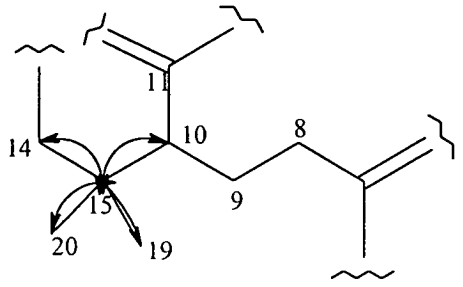
The quaternary ^{13}C signal at 139.9 ppm (C 7) showed a correlation to the methylene ^{13}C signal at 40.9 ppm (C 8) which in turn showed a correlation to the methylene ^{13}C signal at 30.1 ppm (C 9).



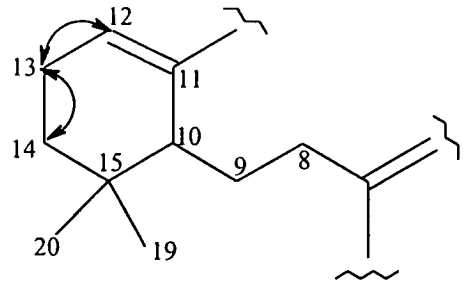
The ^{13}C signal at 30.1 ppm (C 9) also showed a correlation to the methine ^{13}C signal at 49.5 ppm (C 10) which in turn showed correlations to the quaternary ^{13}C signals at 137.0 ppm (C 11) and 33.0 ppm (C 15).



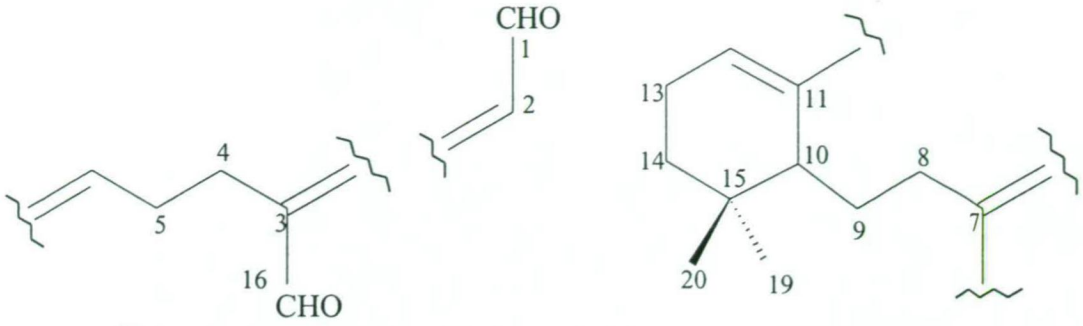
The quaternary ^{13}C signal at 33.0 ppm (C 15) also showed correlations to the methyl ^{13}C signals at 27.7 ppm and 27.9 ppm (C 19/20) and the methylene ^{13}C signal at 32.1 ppm (C 14).



The methylene ^{13}C signal at 32.1 ppm (C 14) also showed a correlation to the methylene ^{13}C signal at 23.5 ppm (C 13) which in turn showed a correlation to the methine ^{13}C signal at 120.5 ppm (C 12).



Hence the results of the INADEQUATE experiment indicated that the following substructures were present in **(8)**:



The two ^{13}C methyl signals at 16.7 ppm and 23.9 ppm were incompletely correlated in the INADEQUATE experiment however, on the basis of the results of the DEPT experiment, were positioned at C 11/7 to fulfil the requirements of these quaternary carbon atoms.

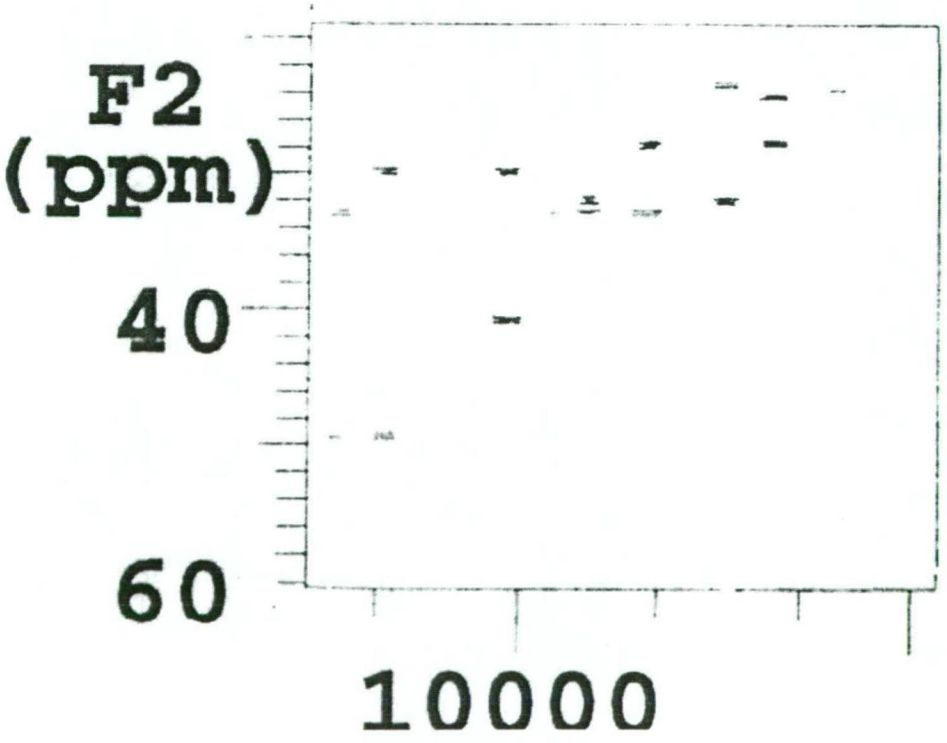


Figure 3.16 Partial INADEQUATE n.m.r. spectrum of (8) (100 MHz, $CDCl_3$)

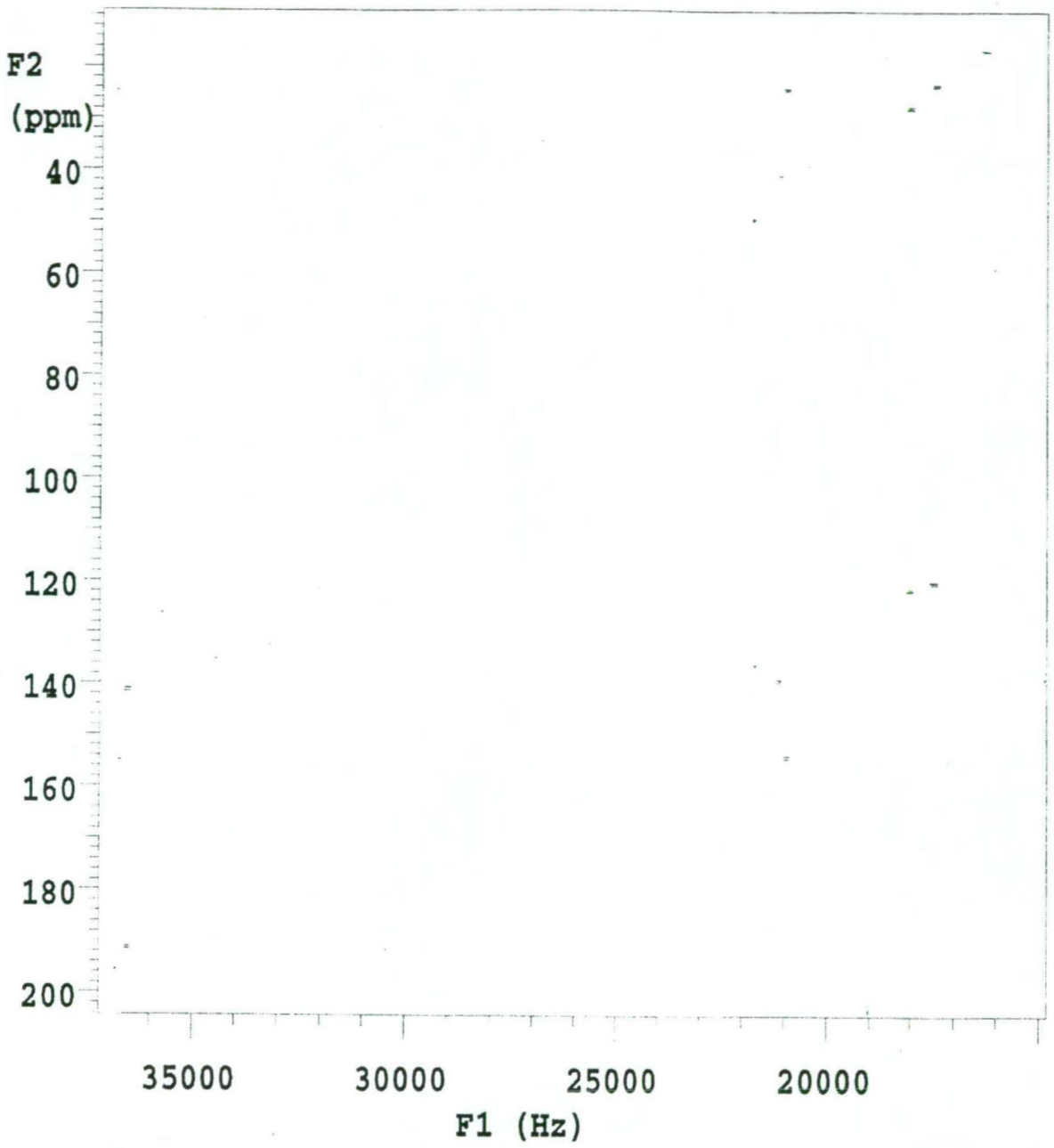
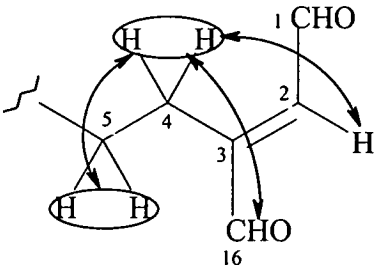


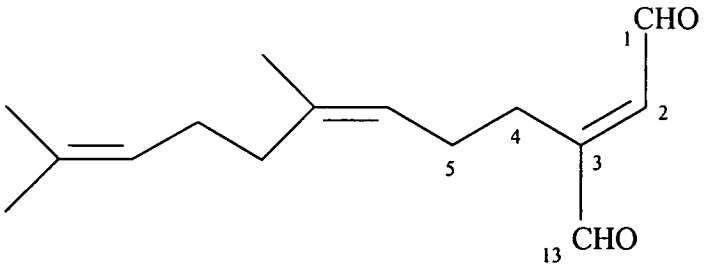
Figure 3.17 Partial INADEQUATE n.m.r. spectrum of (8) (100 MHz, $CDCl_3$)

COSY n.m.r. experiments (Figures 3.18, 3.19) were then conducted on **(8)** to provide information on the ^1H - ^1H couplings within the molecule (Table 3.4).

The COSY n.m.r. experiments showed coupling between the H 1 aldehyde proton at 10.20 ppm and the H 2 proton at 6.51 ppm. This H 2 proton also showed long range coupling (^4J) to the H 4 methylene protons at 2.70 ppm which in turn showed long range coupling (^4J) to the H 16 aldehyde proton at 9.60 ppm. Since both aldehyde functionalities were α,β-unsaturated, the second aldehyde was therefore positioned at C 16.

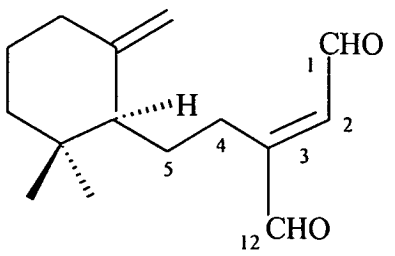


The COSY n.m.r. experiment showed further coupling between the H 4 methylene protons at 2.70 ppm and the H 5 methylene protons at 2.20 ppm. This part of the molecule from C 1 to C 5 is similar to the corresponding part of 3 formyl-7,11-dimethyl-(2*E*, 6*Z*, 10)-dodecatrienal **(9)**; an antifungal compound found in the mandibular gland of the ant *Lasius fuliginosus* Latreille.[8]



(9)

The sesquiterpenoid **(10)** obtained from the tropical green alga *Caulerpa bikinensis*[9] also contains an identical substructure from C 1 to C 5.



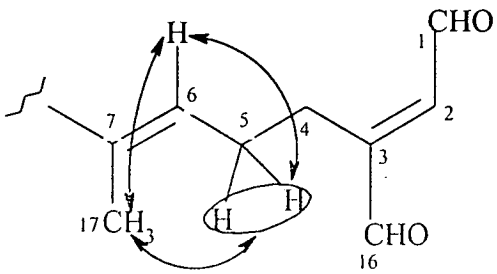
(10)

The relevant ^{1}H and ^{13}C n.m.r. spectral data for **(8)**, **(9)** and **(10)** for the substructure C 1 to C 5 are shown in Table 3.3 and it can be seen they are in close agreement.

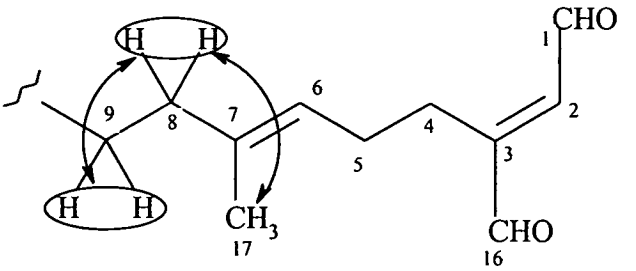
	(8)		**(10)**		**(9)**	
Carbon number	**^{13}C n.m.r.**	**^{1}H n.m.r.**	**^{13}C n.m.r.**	**^{1}H n.m.r.**	**^{13}C n.m.r.**	**^{1}H n.m.r.**
C 1	191.8	10.20	191.6	10.0	191.6	10.20
C 2	141.7	6.51	140.6	5.46	141.1	6.50
C 3	154.6		151.1		154.2	
C 4	24.4	2.70	32.4	2.66/2.43	26.5	2.72
C 5	27.9	2.20	26.2	1.55	27.4	2.21
C 12/13/16	195.5	9.60	195.3	9.60	194.9	9.66

Table 3.3 ^{1}H and ^{13}C n.m.r. data for (8), (9) and (10) in ppm ($CDCl_3$)

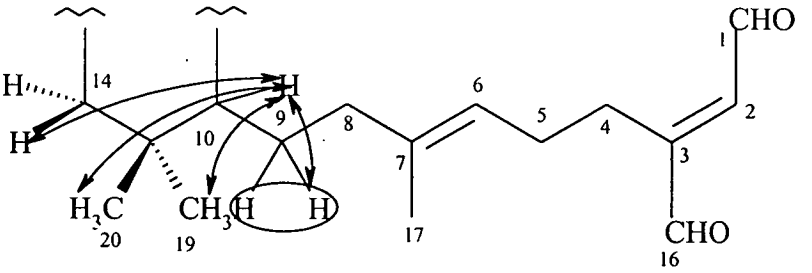
The H 5 protons of **(8)** at 2.20 ppm also showed a COSY correlation to the H 6 methine proton at 5.08 ppm and long range (^{5}J) coupling to the H 17 methyl protons at 1.52 ppm. Furthermore the H 6 methine proton also showed long range (^{4}J) coupling to the H 17 methyl protons at 1.52 ppm.



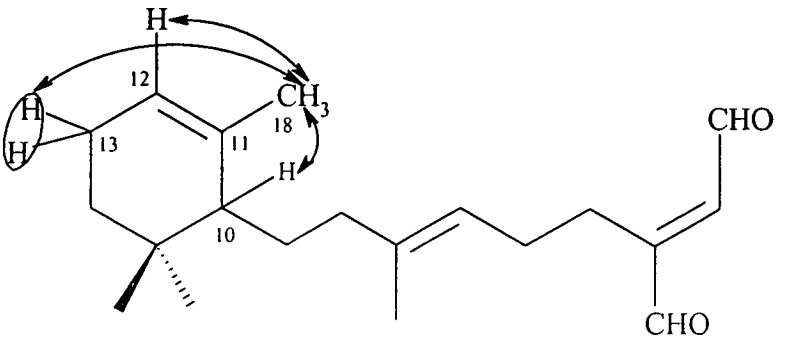
These H 17 methyl protons at 1.52 ppm also showed long range (^4J) coupling to the H 8 protons at 1.96 ppm which in turn showed coupling to the H 9 protons at 1.24 ppm.



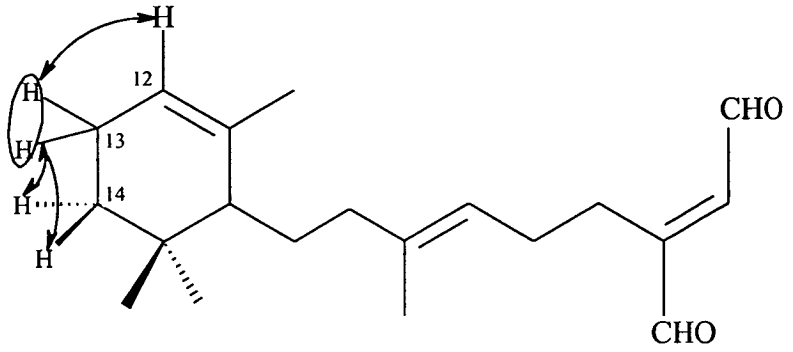
The H 10 proton at 1.38 ppm showed coupling to the H 9 protons at 1.24 ppm, the H 14 proton (*W*) at 1.10 ppm and long range coupling (^4J) to the geminal dimethyl groups (H19/H 20) at 0.85 and 0.90 ppm.



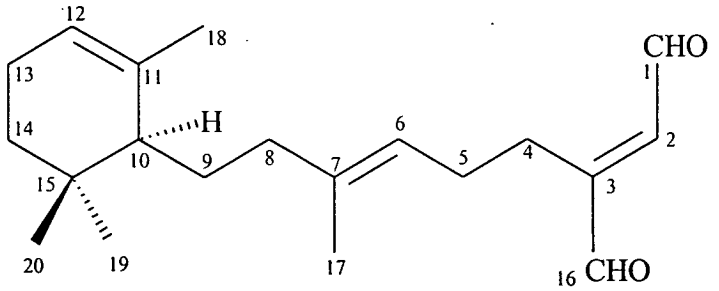
The H 18 methyl protons at 1.60 ppm showed long range coupling (^4J) to the H 12 methine proton at 5.28 ppm, the H 13 methylene protons at 1.96 ppm and the H 10 proton at 1.38 ppm.



The H 12 methine proton at 5.28 ppm also showed coupling to the H 13 protons at 1.96 ppm which in turn were coupled to the H 14 protons at 1.40 and 1.10 ppm.



Therefore, the results of the COSY experiments in conjunction with the INADEQUATE experiment indicated that the structure of **(8)** was:



The stereochemistry of the C 2-C 3 olefin was determined to be *E* by a 2 D NOESY experiment; whilst the stereochemistry of the C 6-C 7 olefin was determined to be *E* by comparison of the shielded ^{13}C value for the vinyl methyl (16.7 ppm) with that of similar compounds **(1)** and **(5)** (Table 3.1) and **(3)**.[6] The absolute stereochemistry of the chiral centre at C 10 was assigned as *S* based on the negative sign for the optical rotation of **(8)**. The reasons for this assignment will be discussed later in this chapter.

Compound **(8)** showed activity in the brine shrimp assay conducted in our laboratory (70% mortality after twenty-four hours, c=0.025 M). The importance of the 1,4-dialdehyde moiety contained in **(8)** will be discussed further in chapter 5.

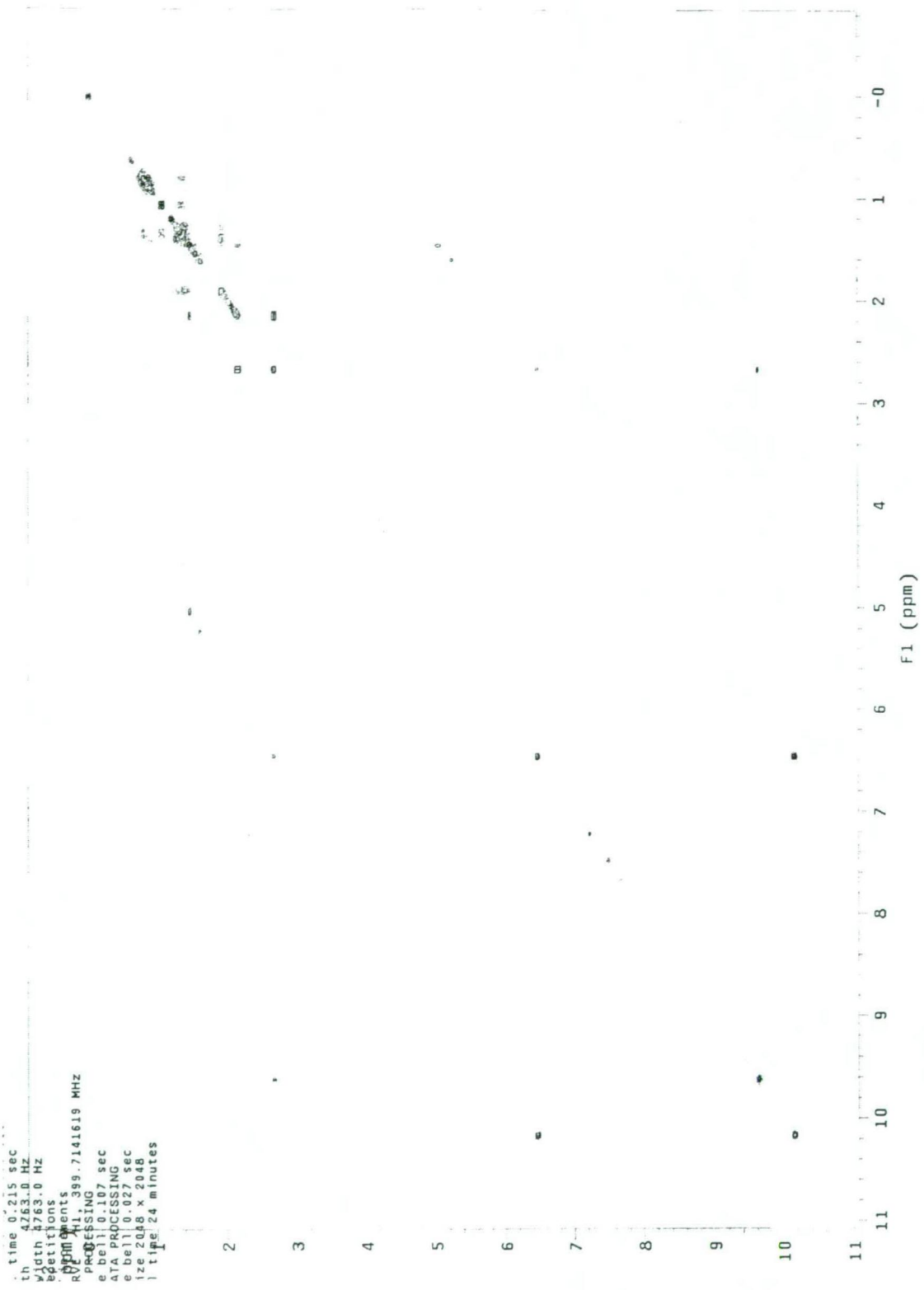


Figure 3.18 COSY n.m.r. spectrum of (8) (100MHz, $CDCl_3$)

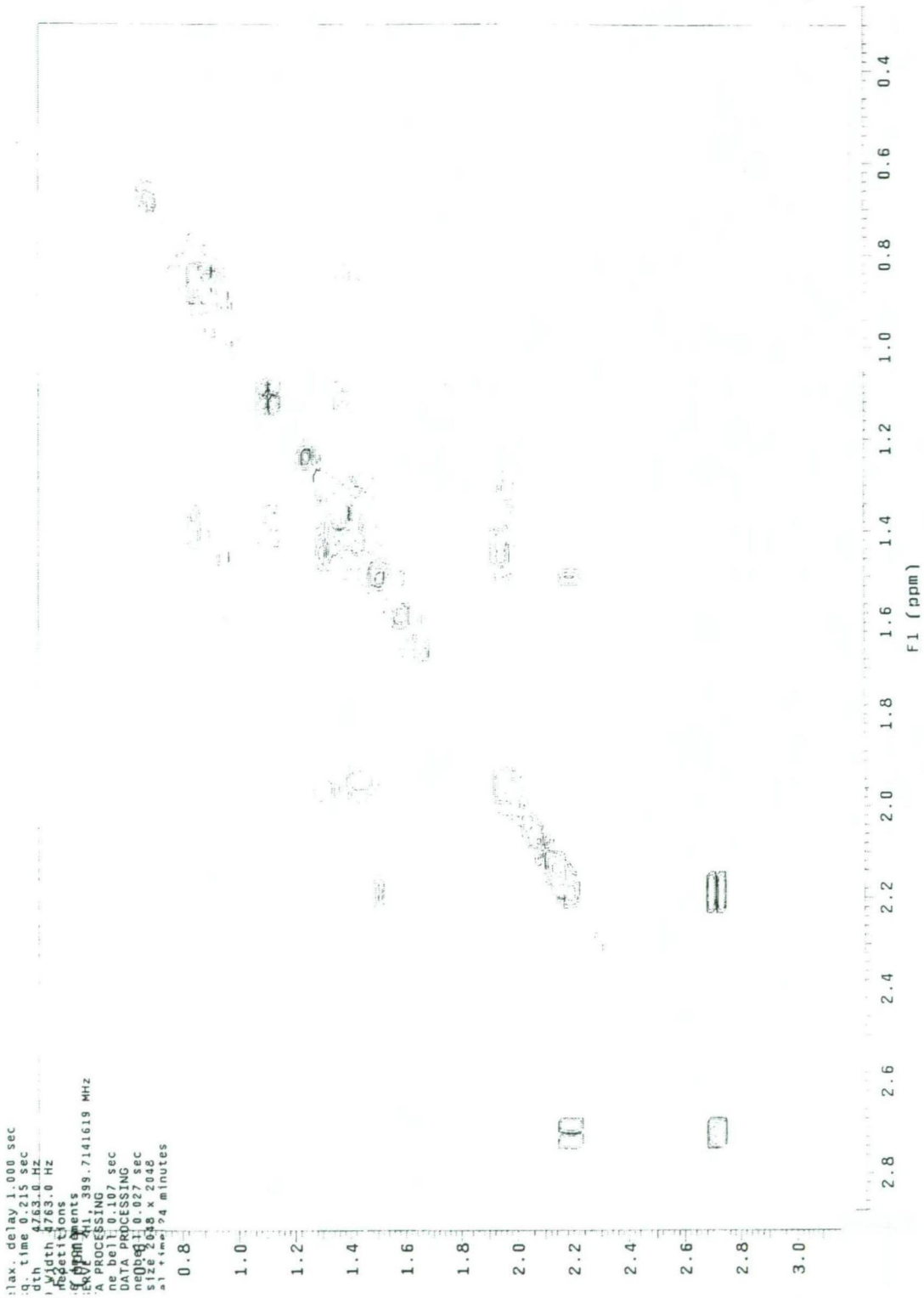


Figure 3.19 COSY n.m.r. spectrum enlargement of (8) (100MHz, $CDCl_3$)

Carbon	^{13}C n.m.r.	^{1}H n.m.r.	COSY	INADEQUATE
1	191.8	10.20 d *J* 7.5 Hz	H 2	C 2
2	141.7	6.51 d *J* 7.5 Hz	H 1, H 4	C 1
3	154.6	----------		C 4, C 16
4	24.4	2.70 t *J* 7.2 Hz	H 2, H 16, H 5	C 3, C 5
5	27.9	2.20 dd *J*14.6, 7.3 Hz	H 4, 6, 8, H 17	C 4
6	121.6	5.08 t *J* 7.5 Hz	H 5, H 17	C 5
7	139.9	----------		C 8
8	40.9	1.96 br s	H 9, H 5	C 7, C 9
9	30.1	1.24 m	H 8, H 10	C 8, C 10
10	49.5	1.38 m	H 9,14,18,19/20	C 9 , C 11, C 15
11	137.0	----------		C 10
12	120.5	5.28 br s	H 13, H 18	C 13
13	23.5	1.96 br s	H 12, 14, 18	C 12, C 14
14	32.1	1.10/1.40 m	H 13, H 10	C 13
15	33.0	----------		C 10,14, C 19/20
16	195.5	9.66 s	H 4	C 3
17	16.7	1.52 s	H 6, H 5	
18	23.9	1.60 s	H 12, 13, 10	
19	27.9	0.90 s	H 10	C 15
20	27.7	0.85 s	H 10	C 15

Table 3.4 GHMQC, COSY and INADEQUATE correlations for (8) ($CDCl_3$)

3.6 Structure elucidation of compound (11)

The next most polar fraction obtained by p.t.l.c. consisted of a mixture of two novel compounds **(11)** and **(12)** which were resolved by reverse phase h.p.l.c. (C 18, 100% MeOH). Compound **(11)** was isolated as a pale yellow oil $[\alpha_D]= -51^0$, (c=5.0 x 10^{-5} M, EtOH) in 0.002% dry algal yield (0.6 mg). By high resolution e.i. mass spectrometry **(11)** was found to have a molecular formula of $C_{24}H_{38}O_4$ (M^+ 390.2770, calc. 390.27701).

The u.v. spectrum (λ_{max} 203 nm, ε=11300, EtOH) and the i.r. spectrum (1744 cm^{-1}), indicated the presence of acetoxy bearing moieties. The e.i. mass spectrum of **(11)** (Figure 3.19) showed the highest molecular ion at 390 and also m/z peaks at 330 and 270; indicating the presence of two acetoxy groups in the compound (loss of acetic acid corresponds to a loss of 60).

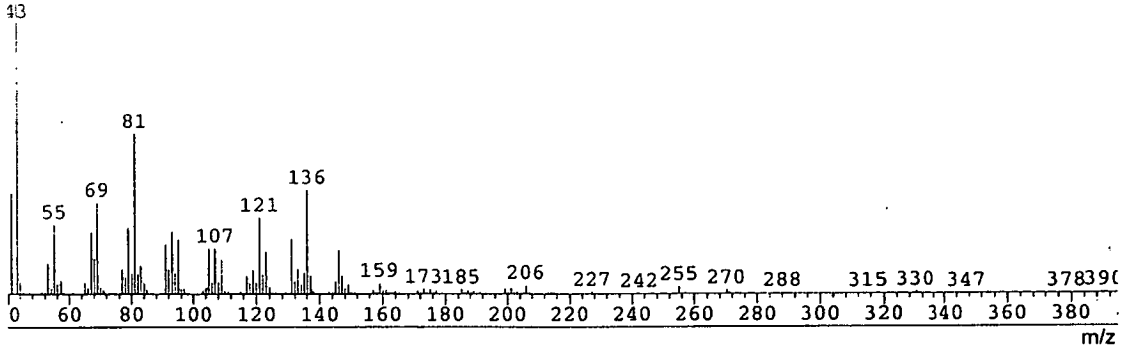


Figure 3.20 E.i mass spectrum of (11)

The ^1H n.m.r. spectrum of **(11)** (Figure 3.21) showed signals at 5.60, 5.28 and 5.08 ppm characteristic of protons attached to carbon-carbon double bonds, and a doublet

at 4.63 ppm and a singlet at 4.54 ppm characteristic of protons attached to an oxgenated carbon atom. The ^{1}H n.m.r. signal at 4.63 ppm showed coupling to the methine proton at 5.60 ppm (t *J* 6.9 Hz, H 2).

The remainder of the spectrum between 0.80 ppm and 2.30 ppm closely resembled that of **(5)**, containing two sharp singlets at 2.04 and 2.07 ppm typical of acetoxy methyl groups, signals in the 1.5-1.7 ppm and 0.8-0.95 ppm regions characteristic of methyl groups and signals in the saturated aliphatic region between 1.0 and 2.30 ppm.

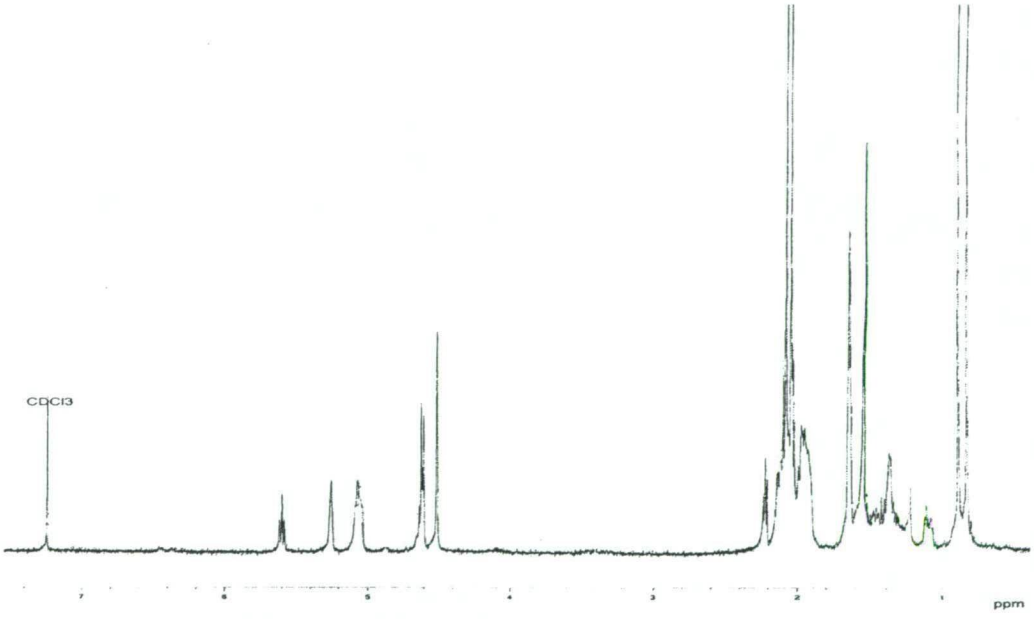


Figure 3.21 ^{1}H n.m.r. spectrum of (11) (400 MHz, $CDCl_3$)

The ^{13}C n.m.r. spectrum (Figure 3.22) of **(11)** contained twenty-four resonances; two signals characteristic of carbonyl carbons at 171.1 and 170.8 ppm, six signals in the vinyl region between 140 and 120 ppm and two signals at 60.6 and 67.2 ppm which were characteristic of acetoxy methylene moieties. The position of the remaining 14 signals

between 50 and 16 ppm were almost identical to the corresponding carbon resonances in **(5)** and **(8)** (Table 3.1).

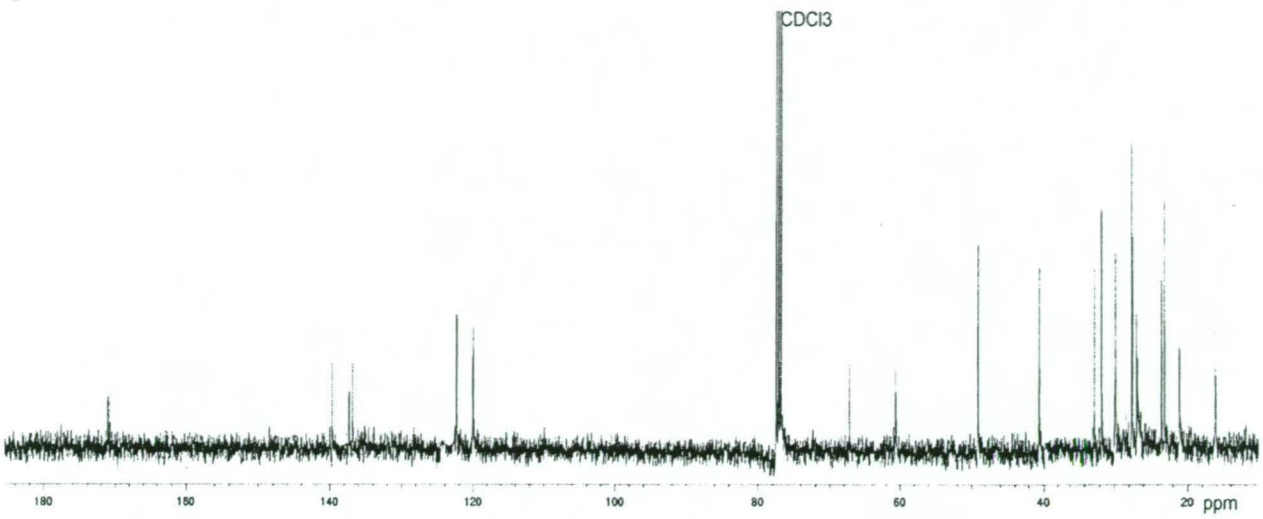


Figure 3.22 ^{13}C n.m.r. spectrum of (11) (100 MHz, $CDCl_3$)

The multiplicity of the protonated carbons of **(11)** was determined by a DEPT n.m.r. experiment (Figure 3.23); indicating that **(11)** possessed four methine, eight methylene and six methyl groups. The six remaining ^{13}C resonances at 171.1, 170.8, 139.7, 137.3, 136.9 and 32.7 ppm were therefore attributed to quaternary carbon atoms (Table 3.1).

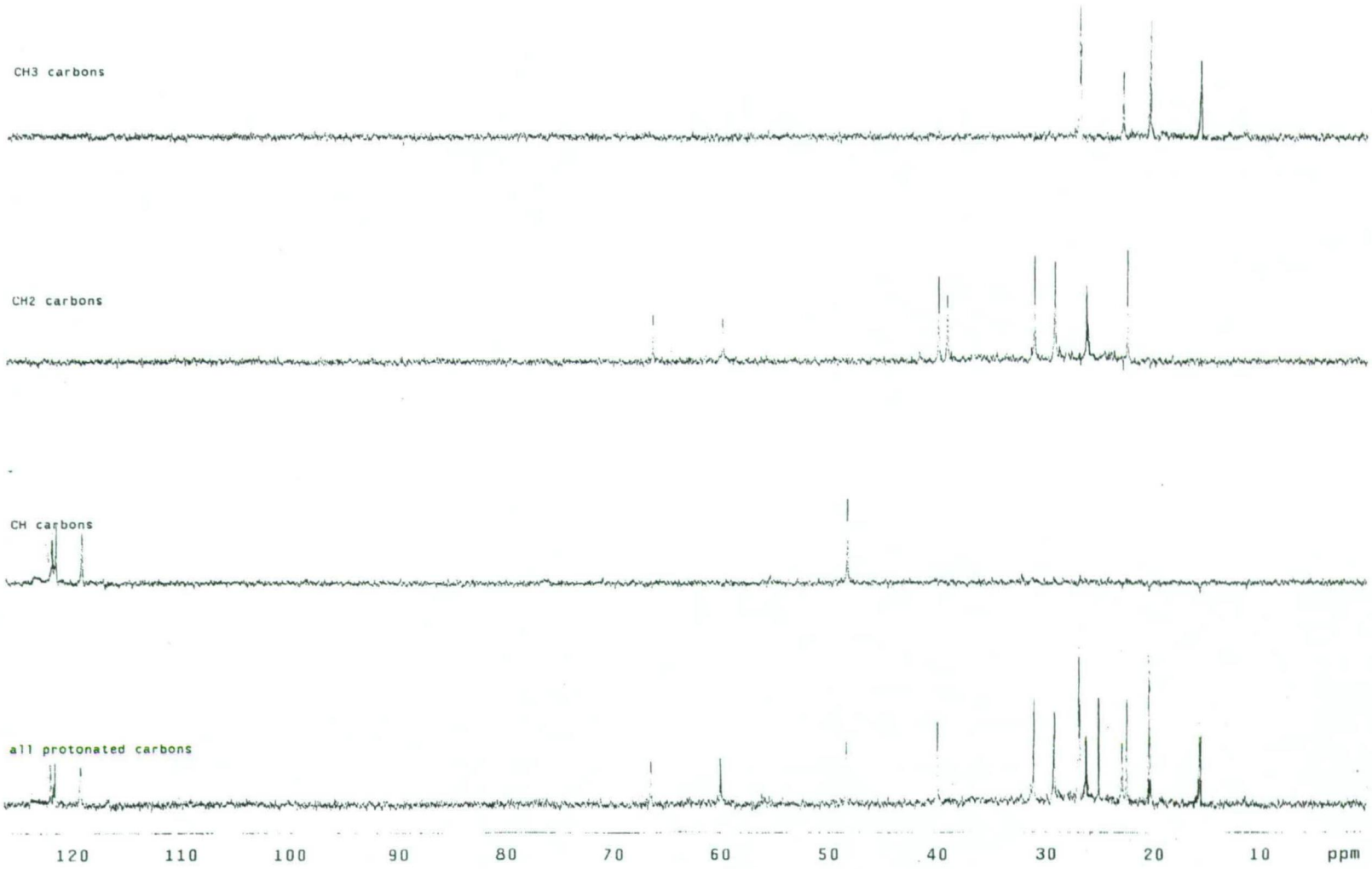


Figure 3.23 DEPT n.m.r. spectrum of (11) (100MHz, $CDCl_3$)

A GHMQC n.m.r. experiment (Figure 3.24) provided the ^{13}C-^{1}H connections in **(11)**, (Table 3.6) whilst analysis of a COSY n.m.r. experiment determined the ^{1}H-^{1}H couplings within the molecule (Table 3.6).

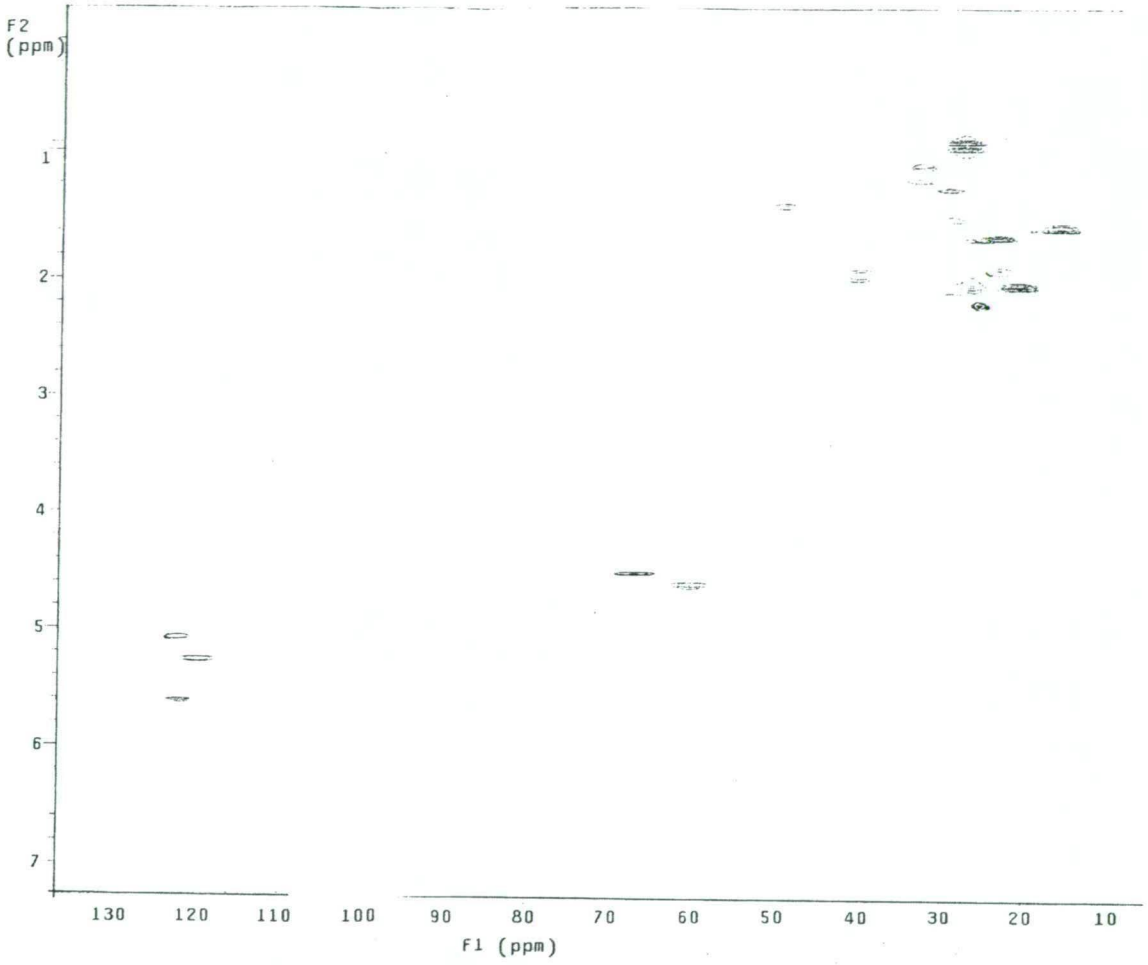
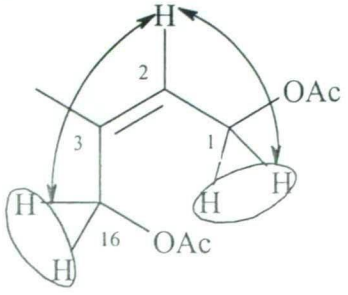
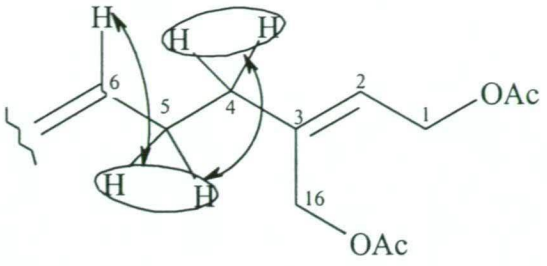


Figure 3.24 GHMQC spectrum of (11) (100MHz, $CDCl_3$)

The COSY n.m.r. experiment (Figure 3.25) showed coupling between the H 1 methylene protons at 4.63 ppm and the H 2 methine proton at 5.60 ppm. This H 2 proton also showed long-range coupling (^{4}J) to the H 16 methylene protons at 4.54 ppm.



The H 4 methylene protons at 2.25 ppm showed coupling to the H 5 protons at 2.05 ppm which in turn showed coupling to the H 6 methine proton at 5.08 ppm.



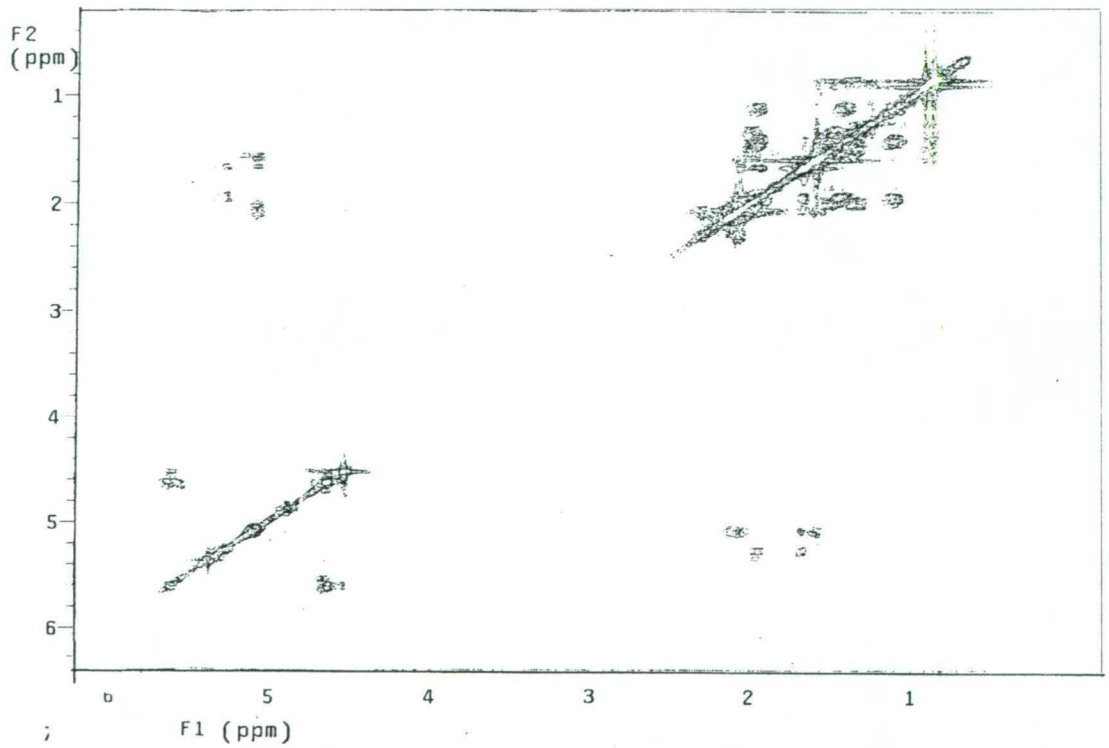
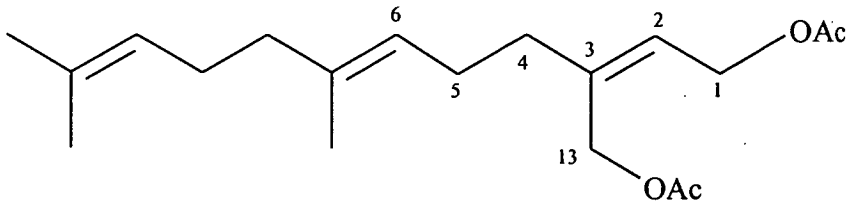


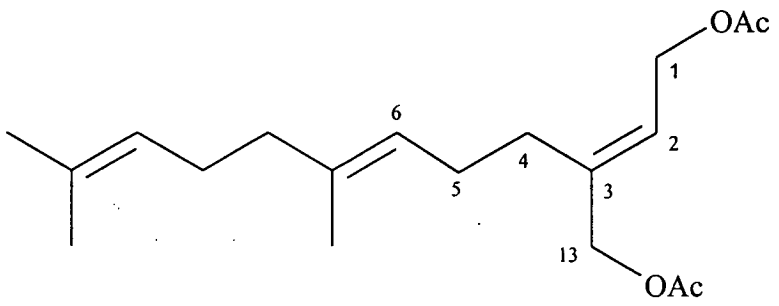
Figure 3.25 COSY n.m.r. spectrum of (11) (400MHz, $CDCl_3$)

The structural moiety C 1 to C 6 of **(11)** is identical to the corresponding moiety of **(13)** which was obtained by reduction and subsequent acetylation of flexilin in 1978 by the Blackman research group.[2] The ^{1}H n.m.r. signals due to allylic AcO-CH_2 groups at 4.55 ppm (d, *J* 7 Hz, H 1) and 4.47 ppm (s, H 13) are almost identical to the corresponding signals in **(11)**.



(13)

A similar compound **(14)** was isolated in 1990 by Wright and Coll off the North Queensland coast from the tropical green alga *Chlorodesmis fastigiata*[10]; didehydrotrifarin **(2)** was also isolated from this collection of *C. fastigiata.*



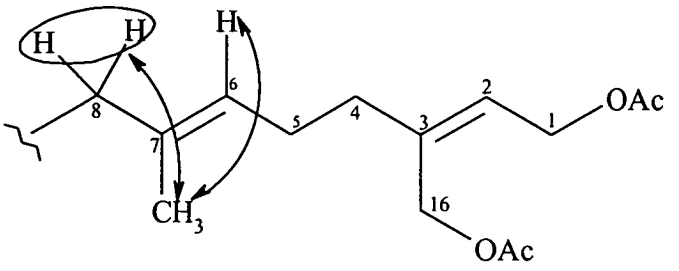
(14)

The stereochemistry of the C 2-C 3 double bond in **(14)** was determined to be *E* on the basis of n.O.e experiments. Hence the stereochemistry of the C2-C 3 double bond in **(11)** was determined to be *Z* since its ^{1}H n.m.r. spectral data (the difference between the corresponding resonances) are in closer agreement with **(13)** than **(14)** (Table 3.5). A secondary metabolite containing an identical C 1 –C 5 moiety to **(14)** will be discussed in Chapter 4.

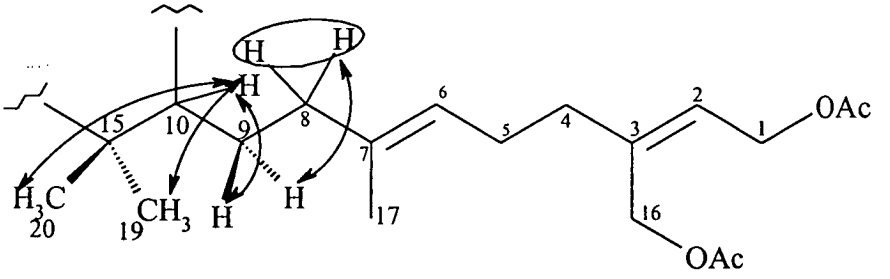
Carbon no.	(13) ppm	(14) ppm	(11) ppm
1	4.55	4.66	4.63
2		5.56	5.60
13/17	4.47	4.63	4.54

Table 3.5 ^{1}H n.m.r. data for (13), (14) and (11) ($CDCl_3$)

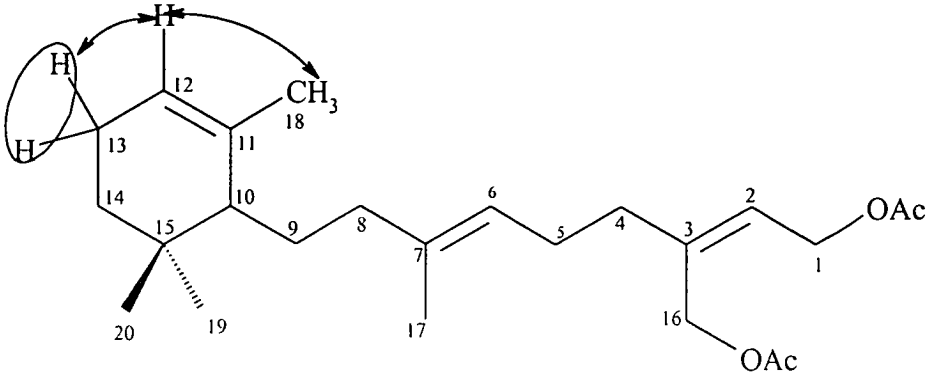
The COSY connections for the remainder of **(11)** (Table 3.6) are as follows. The H 6 methine proton at 5.08 ppm also showed long range (^4J) coupling to the H 17 methyl protons at 1.58 ppm which in turn showed long range coupling (^4J) to the H 8 methylene protons at 1.98 ppm.



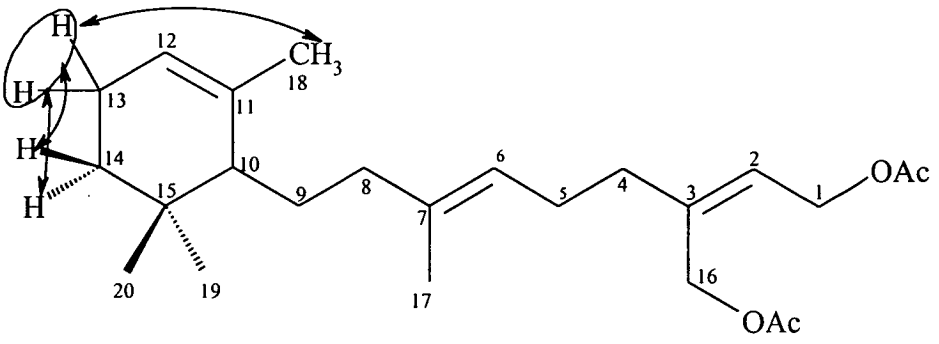
These H 8 protons also showed coupling to the H 9 proton at 1.28. The H 9 protons in turn showed coupling to the H 10 methine proton at 1.40 ppm which in turn showed long range coupling (^4J) to the geminal dimethyl groups (H 19/20).



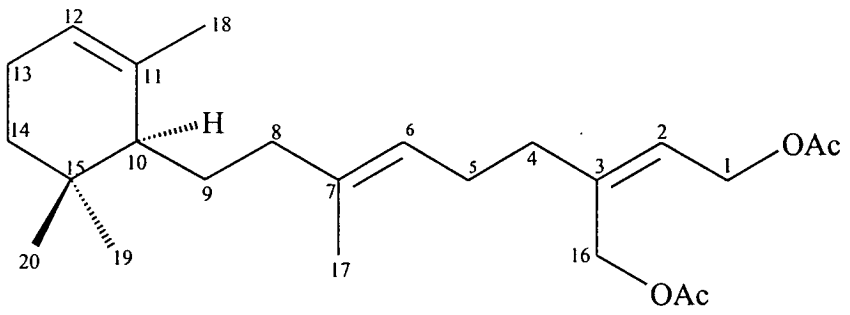
The H 12 methine proton at 5.28 ppm showed coupling to the H 13 methylene protons at 1.98 ppm and long range coupling (^4J) to the H 18 methyl protons at 1.64 ppm.



These H 18 methyl protons also showed long-range coupling to the H 13 methylene protons at 1.95 ppmwhich in turn showed coupling to the H 14 methylene protons at 1.38 ppm and 1.10 ppm.



Thus the spectral evidence presented indicates that the structure of **(11)** is as shown below:



(11)

The absolute stereochemistry of the chiral centre at C 10 was assigned as *S* based on the negative sign for the optical rotation of **(11)**. The reasons for this assignment will be discussed later in this chapter.

Carbon no.	^{13}C n.m.r.	^{1}H n.m.r.	COSY
1	60.6 (t)	4.63 t *J* 6.9 Hz	H 2
2	122.4 (d)	5.60 t *J* 6.9 Hz	H 1, H 16
3	139.7 (s)	----------	
4	25.8 (t)	2.25 m	H 5
5	27.0 (t)	2.05 m	H 4
6	122.8 (d)	5.08 br s	H 5, H 17
7	136.9 (s)	----------	
8	40.7 (t)	1.98 br s	H 9
9	29.9 (t)	1.48 m 1.28 m	H 8, H 10
10	49.1 (d)	1.40 m	H 9, H 19/20
11	137.3 (s)	----------	
12	120.0 (d)	5.28 br s	H 13, H 18
13	23.2 (t)	1.98 br s	H 12, H 14
14	31.7 (t)	1.38 m 1.10 m	H 13, H 13, H 10
15	32.7 (s)	----------	
16	67.2 (s)	4.54 s	H 4
17	16.2 (q)	1.58 s	H 6, H 8
18	23.6 (q)	1.64 d *J* 1.6 Hz	H 12, H13
19	27.6 (q)	0.90 s	H 10, H 9
20	27.8 (q)	0.84 s	H 10, H 9, H 14b
$O\underline{C}OCH_3$	171.1 (s) 170.8 (s)		
$OCO\underline{C}H_3$	2x 21.1 (q)	2.07 s, 2.04 s	

Table 3.6 ^{1}H, ^{13}C and COSY n.m.r data for (11) (ppm, $CDCl_3$)

3.7 Structure elucidation of (12)

Compound **(12)** was isolated as a pale yellow oil $[\alpha_D]=-49^0$, (c=5.0 x 10^{-5}M, EtOH) in 0.001% dry algal yield. By high resolution e.i. mass spectrometry **(12)** was found to have a molecular formula of $C_{22}H_{34}O_3$ (M^+ 346.25135, calc. 346.25080). The u.v. spectrum (λ_{max}=203nm, ε=6055, EtOH) and the i.r. spectrum (1742, 1691 cm^{-1}) indicated the presence of acetoxy and α,β-unsaturated aldehyde moieties.

The e.i. mass spectrum of **(12)** (Figure 3.26) showed a loss of 60 mass units (from 346 to 286) corresponding to the loss of acetic acid, suggesting the presence of an acetoxy group. The e.i. mass spectrum of **(12)** also showed a very similar fragmentation pattern to that of **(8)** and **(11)** consistent with the presence of a substituted dimethyl-cyclohexene moiety.

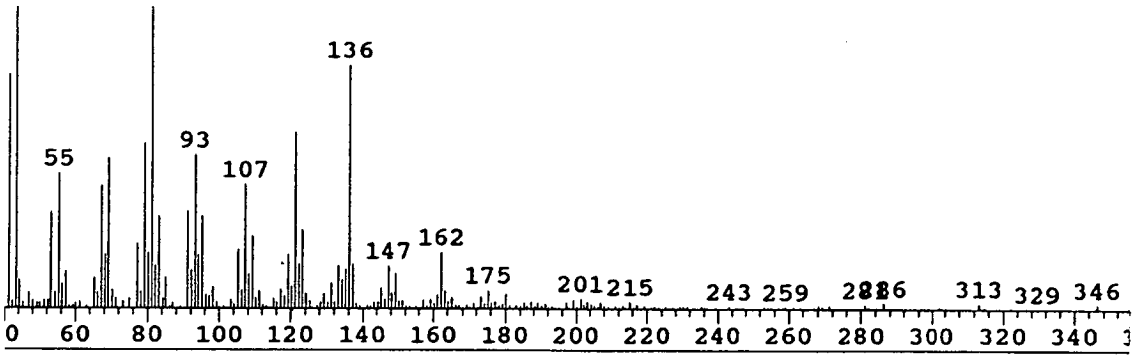


Figure 3.26 E.i. mass spectrum of (12)

The ^{1}H n.m.r. spectrum of **(12)** (Figure 3.27) included signals for an aldehyde proton at 9.41 ppm, an olefinic proton at 6.44 ppm (d, *J* 6.0 Hz) which showed coupling to an allylic methylene group at 4.88 ppm (d, *J* 6.0 Hz) and an acetoxy methyl group at 2.12 ppm. The remainder of the spectrum between 0.80 ppm and 2.30 ppm closely resembled those of (5), (8) and (11); containing signals in the 1.5-1.7 ppm and 0.8-0.95

ppm regions characteristic of methyl groups and signals in the saturated aliphatic region between 1.0 and 2.30 ppm.

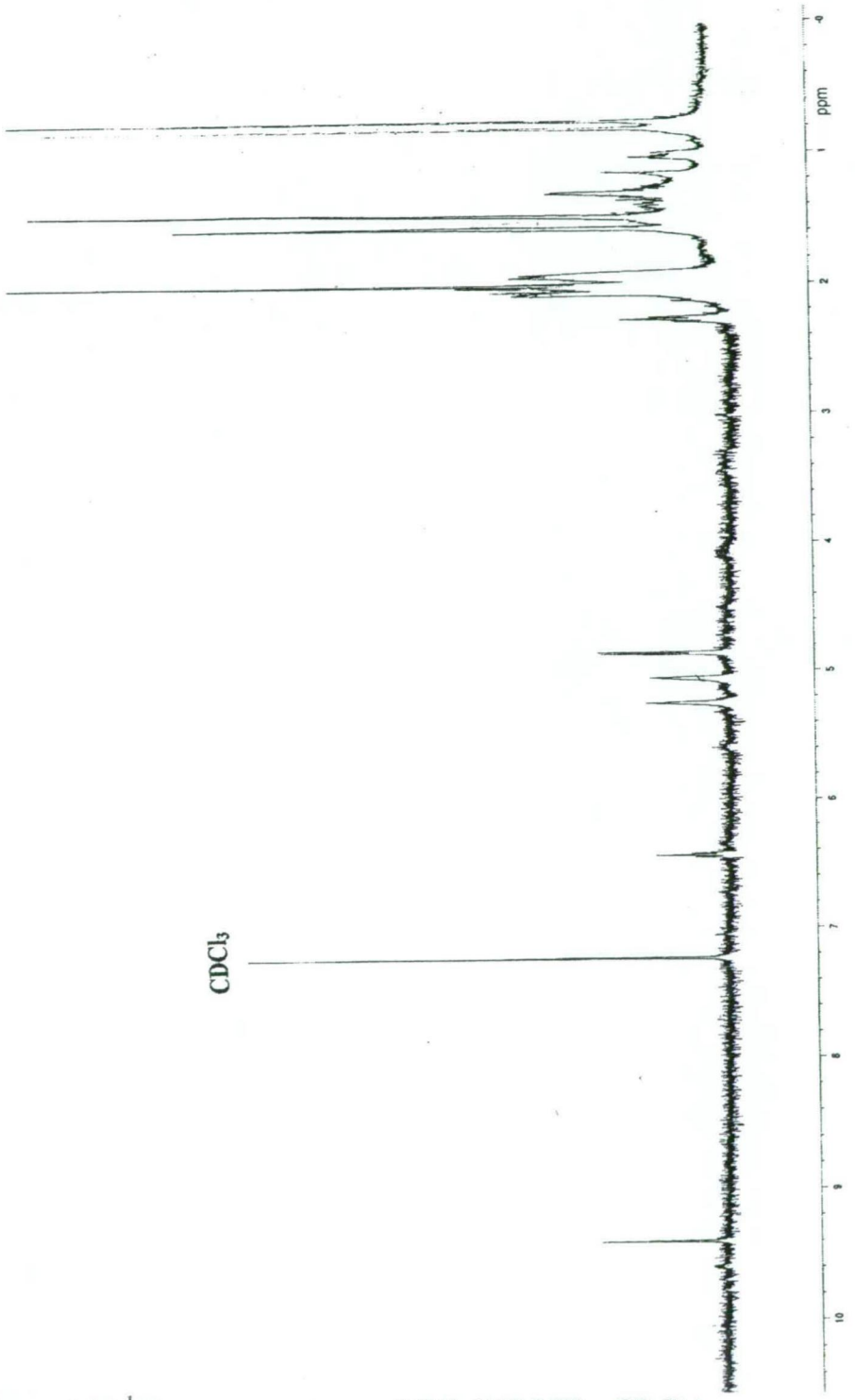


Figure 3.27 ^{1}H n.m.r. spectrum of (12) (400 MHz, $CDCl_3$)

Due to the small amount of compound **(12)** isolated (0.4 mg) the remaining n.m.r. experiments were carried out at 400 MHz using a nanoprobe. The ^{13}C n.m.r. spectrum (Figure 3.28) of **(12)** contained twenty-two resonances; a signal at 194.0 ppm characteristic of an aldehydic carbon, a signal characteristic of a carbonyl carbon at 171.0 ppm, six signals in the vinyl region between 147 and 120 ppm and a signal at 60.9 ppm typical of an methylene attached to an acetoxy moiety. The positions of the remaining 14 signals between 50 and 16 ppm were almost identical to the corresponding carbon resonances in **(5)** and **(8)** (Table 3.1). The multiplicities of the protonated carbons of **(12)** were determined by a DEPT experiment (Figure 3.29); indicating that **(12)** possessed five methine, seven methylene and five methyl groups. The five remaining ^{13}C resonances at 171.0, 144.5, 137.6, 136.8 and 32.7 ppm were therefore attributed to quaternary carbon atoms (Table 3.1).

A HETCOR experiment provided information on the ^{13}C-^{1}H connections in **(12)**, (Table 3.8) whilst analysis of COSY n.m.r. experiments (short and long range) provided information on the ^{1}H-^{1}H couplings within the molecule (Table 3.8).

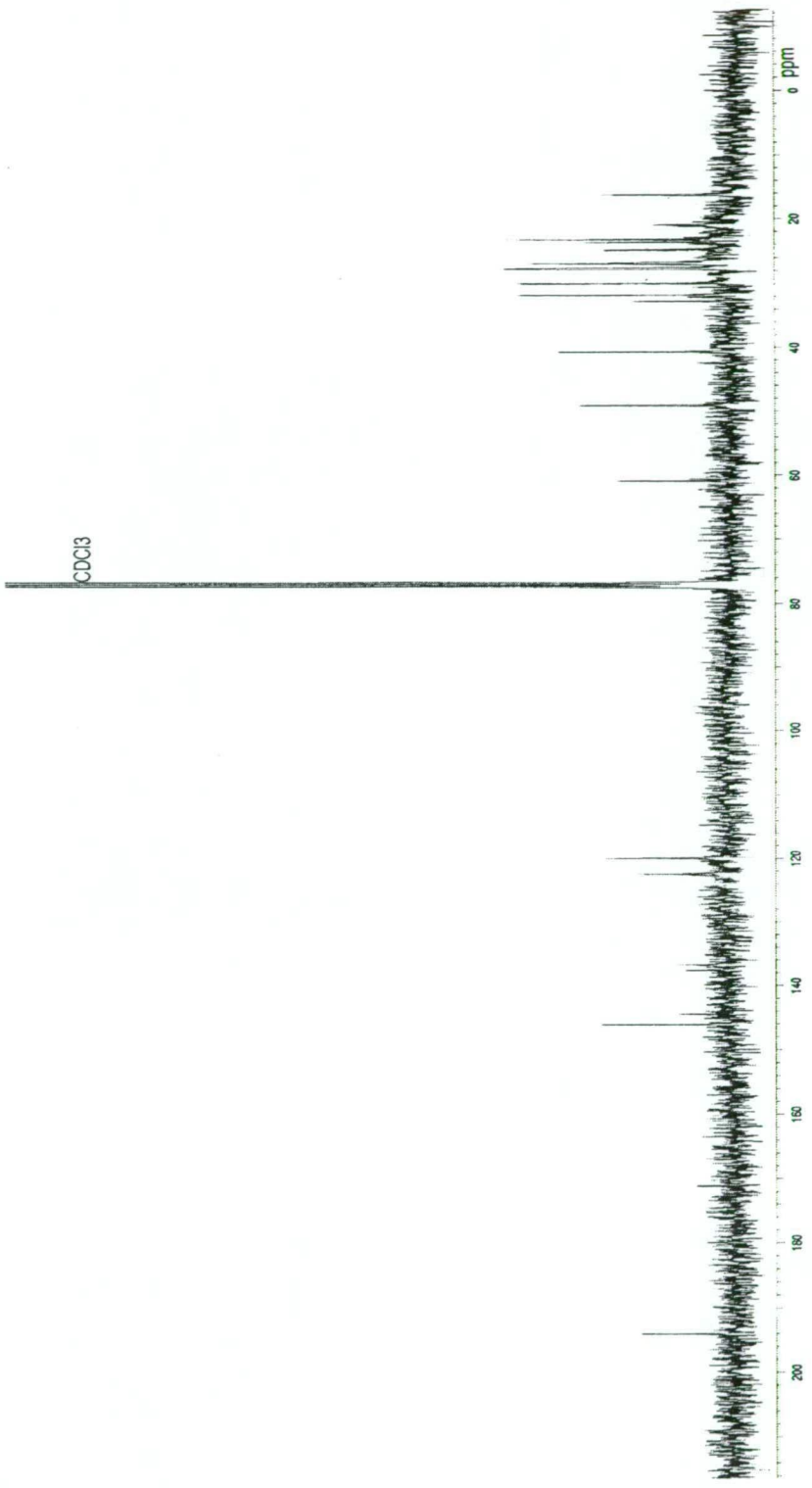


Figure 3.28 ^{13}C n.m.r. spectrum of (12) (100 MHz, $CDCl_3$)

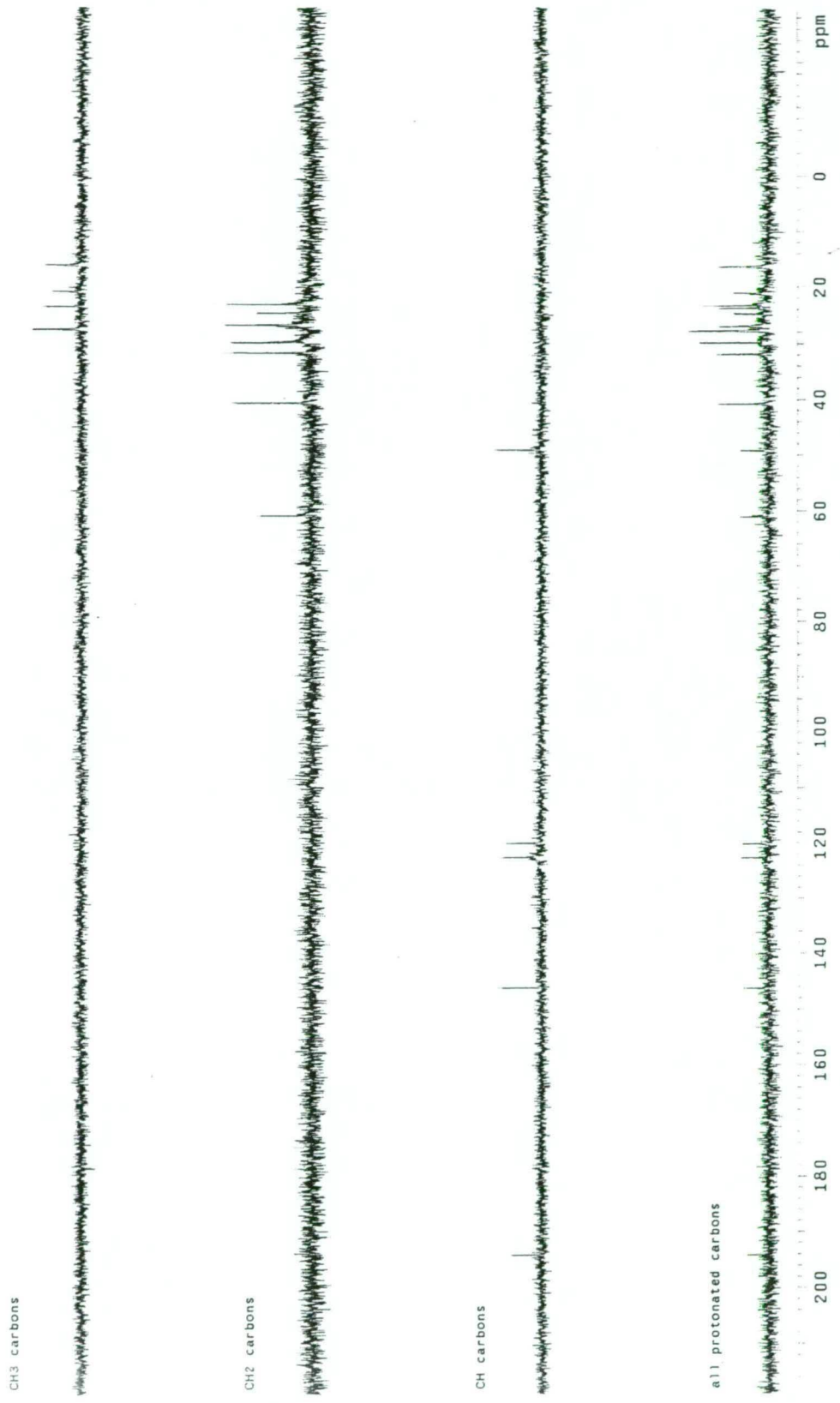
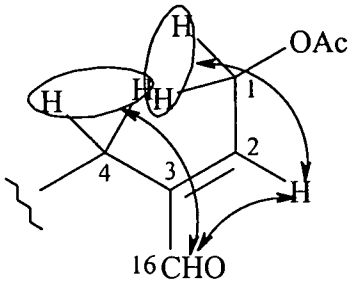
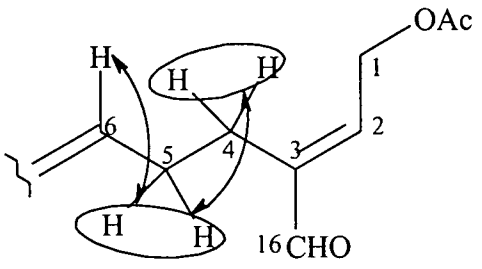


Figure 3.29 DEPT n.m.r. spectrum of (12) (100 MHz, $CDCl_3$)

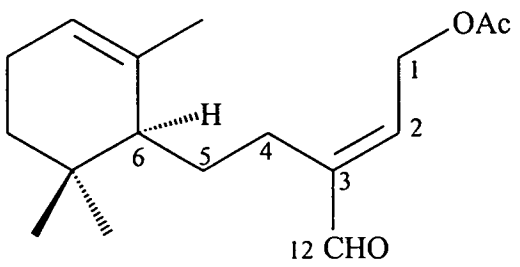
The COSY n.m.r. experiments (Figures 3.30, 3.31) showed coupling between the H 1 methylene protons at 4.88 ppm and the H 2 methine proton at 6.44 ppm. The H 1 methylene protons also showed long-range coupling (^5J) to the H 4 methylene protons at 2.27 ppm. These H 4 methylene protons also showed long-range coupling (^4J) to the H 16 aldehyde proton at 9.41 ppm.



Furthermore the H 4 methylene protons at 2.27 ppm showed coupling to the H 5 methylene protons at 2.05 ppm which in turn showed coupling to the H 6 methine proton at 5.06 ppm.



The substructure C 1 to C 5 of **(12)** is identical to the corresponding substructure of **(7)** isolated from *C. flexilis* (Lamx.) var. *muelleri* (Sond) (Wom.)[4] and it can be seen that the corresponding ^1H and ^{13}C n.m.r. values are in close agreement (Table 3.7).

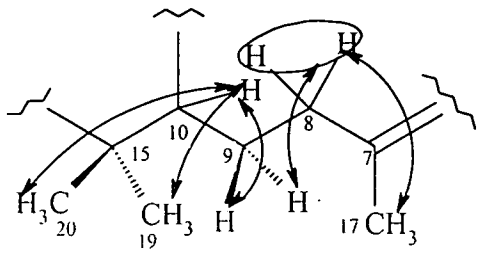


(7) (12)

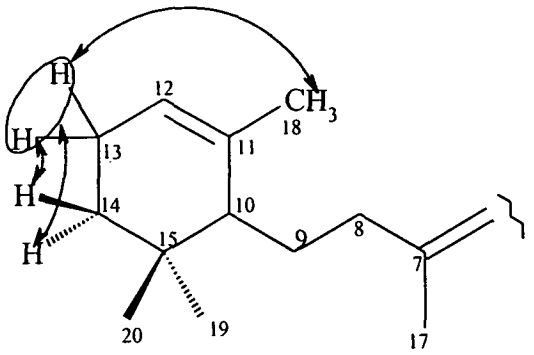
Carbon #	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	60.6	4.90	60.9	4.88
2	145.4	6.41	146.2	6.44
3	146.1	-------	144.5	------
4	23.1		24.8	2.27
5	25.1		26.9	2.05
16/12	194.2	9.41	194.0	9.41
$OCOCH_3$	170.7	2.11	171.0	2.12

Table 3.7 ^{1}H and ^{13}C n.m.r. data for C 1 to C 5 of (7) and (12) ($CDCl_3$)

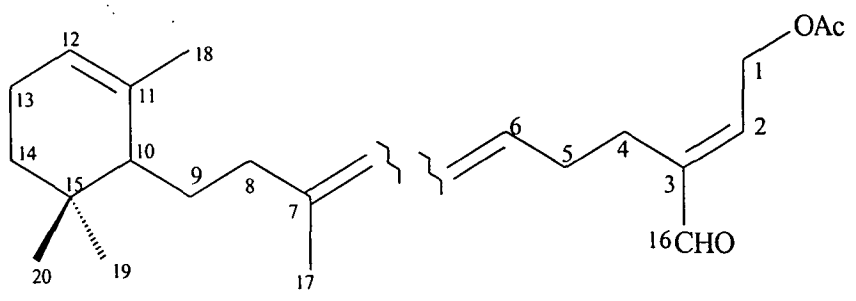
The COSY connections for the remainder of **(12)** (Table 3.8) are as follows. The H 17 methyl protons at 1.58 ppm showed long-range coupling (^{4}J) to the H 8 methylene protons at 1.98 ppm. These H 8 protons also showed coupling to one of the H 9 protons. The H 9 protons in turn showed coupling to the H 10 methine proton at 1.40 ppm. This H 10 proton also showed long-range coupling (^{4}J) to the geminal dimethyl groups (H 19/H 20).



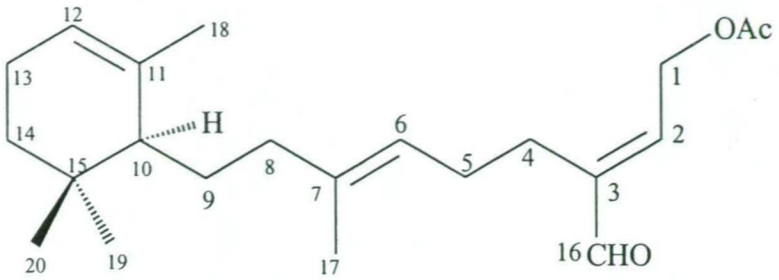
The H 18 methyl protons at 1.68 ppm showed long-range coupling (^5J) to the H 13 protons at 1.95 ppm, which in turn showed coupling to the H 14 protons at 1.38 ppm and 1.10 ppm.



Thus the evidence presented so far indicates that two substructures are present in **(12)**:



Whilst the COSY n.m.r. experiments did not show long range coupling between the H 6 methine proton and the H 17 methyl protons it seems reasonable to link the two substructures together on biosynthetic grounds. The absolute stereochemistry of the chiral centre at C 10 was assigned as *S* based on the negative sign for the optical rotation of **(12)** (the reasons for this assignment will be discussed in the next section). The configuration of the C 2-C 3 olefin was assigned as *E*, based on the chemical shift of the aldehyde proton, 9.41 ppm, corresponding to the slightly shielded values recognised for the *E* configuration (*E* = 9.3-9.4, *Z* = 10.0-10.1 ppm)[11] of this functional group. The evidence presented for metabolite **(12)** is therefore consistent with the structure below:



(12)

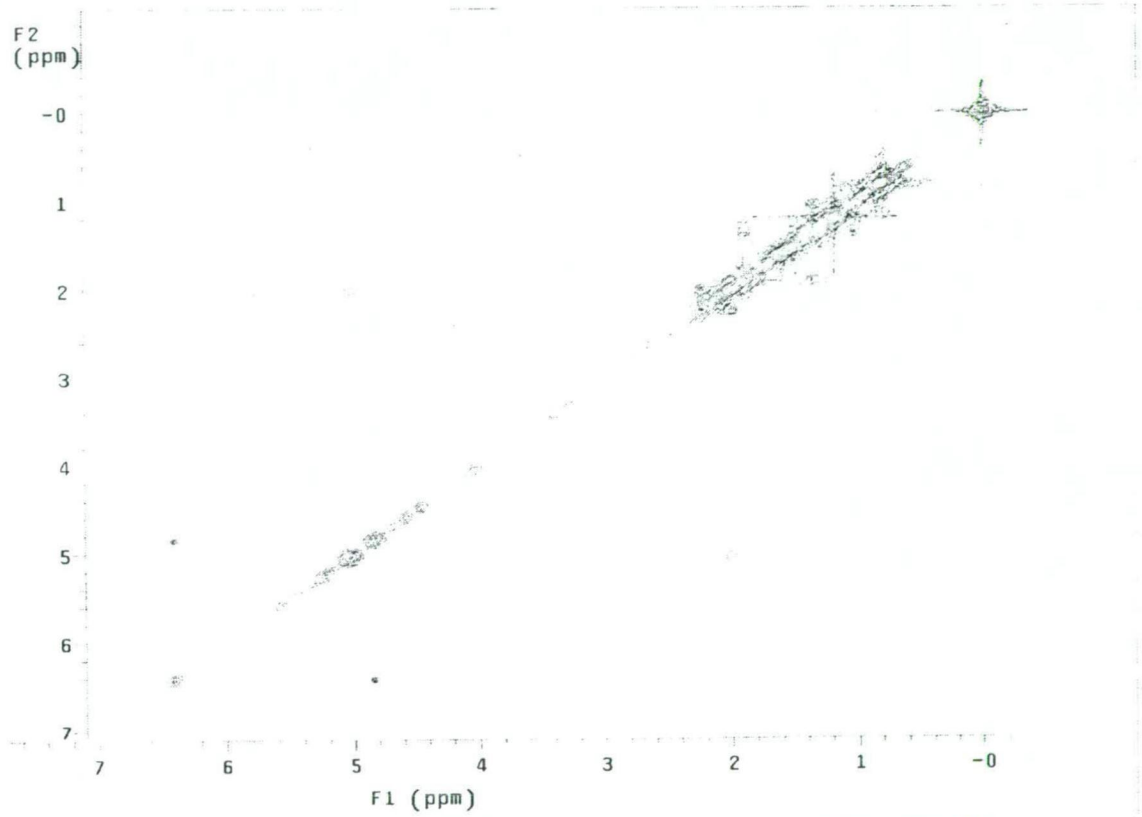


Figure 3.30 COSY n.m.r. spectrum of (12) (100 MHz, $CDCl_3$)

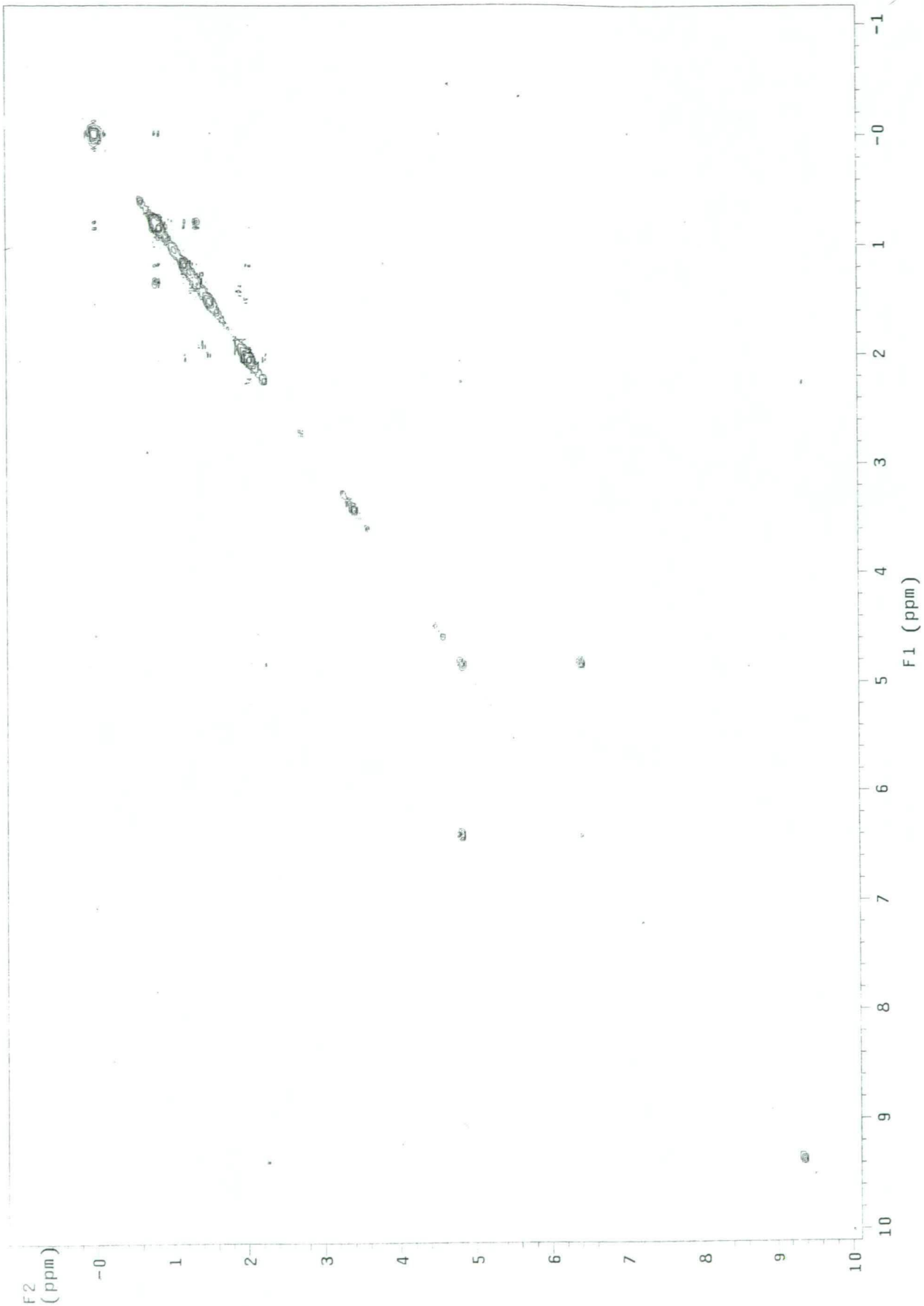


Figure 3.31 COSY n.m.r. long range spectrum of (12)

It is interesting to note that a secondary metabolite with a matching mass spectral profile (Figure 3.32) was identified in both the extract and a p.t.l.c. fraction of *C. brownii* (unbranched). This will be discussed further in Chapter 4.

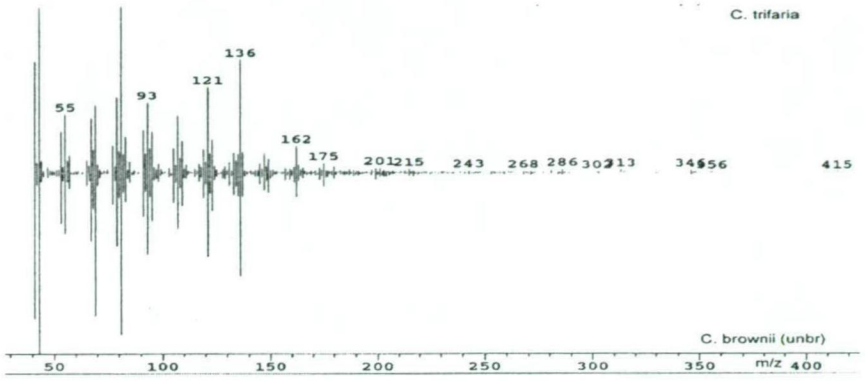


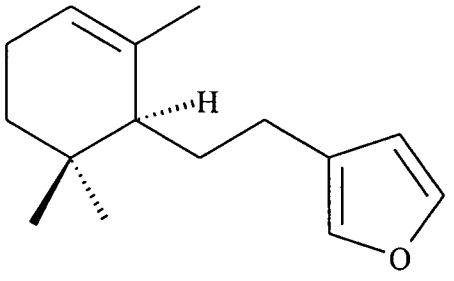
Figure 3.32 E.i. mass spectral match of metabolites from *C. trifaria* and *C. brownii*

Carbon	^{13}C (ppm)	^{1}H (ppm)	COSY
1	60.9	4.88 d *J* 6.0 Hz	H 2, H 4
2	146.2	6.44 t *J* 6.0 Hz	H 1
3	144.5	-----	
4	24.8	2.27 t *J* 7.6 Hz	H 1, H 16, H 5
5	26.9	2.05 t *J* 7.6 Hz	H 4, H 6
6	122.5	5.06 br s	
7	137.6	------	
8	40.7	1.98 m	H 9
9	29.9		H 8, H 10
10	49.2	1.40 m	H 9, H 19, H 20
11	136.8	---------	
12	120.0	5.28 br s	
13	23.3	1.95 br s	H 12, H 14
14	31.8	1.38 m 1.10 m	H 13 H 13, H 10
15	32.8	--------	
16	194.0	9.41 s	H 4
17	16.3	1.58 s	H 6, H 8
18	23.7	1.68 d *J* 1.6 Hz	H 12, H 13
19	27.7	0.90 s	H 10, H 9
20	27.8	0.84 s	H 10, H 9, H 14
$O\underline{C}OCH_3$	171.0	-------	
$OCO\underline{C}H_3$	21.0	2.12 s	

Table 3.8 ^{1}H, ^{13}C and COSY n.m.r data for (12) (ppm, $CDCl_3$)

3.8 Absolute stereochemistry of compounds (5), (8), (11) and (12)

Carman[12] has shown that for a large number of labdane diterpenes in which an asymmetric ring structure is separated by two methylene groups from an asymmetric centre in a chain, the optical rotation of the two portions is approximately additive, provided no interaction occurs between these two centres. In the case of **(5)**, **(8)**, **(11)** and **(12)** where the chain contains no asymmetric centre, the optical rotation values should be of similar magnitude and sign. This is the case with **(5)** $[M_D]$=-290^0, **(8)** $[M_D]$= -214^0, **(11)** $[M_D]$=-200^0 and **(12)** $[M_D]$=-170^0. Comparison of these values with those of **(6)** and **(7)** $[M_D]$=-151^0 and $[M_D]$=-164^0 respectively and that of pallescensin-1 **(15)** $[M_D]$=-191^0 whose absolute configuration has been determined as *S* suggest that **(5)**, **(8)**, **(11)**, **(12)** and **(15)** all have the same absolute configuration.



(15)

3.9 Isolation of compounds (4) and (16)

The next most polar fraction yielded **(4)** as a minor component (0.002% dry algal weight) after preparative t.l.c. The compound was found to have a molecular weight of 374 by l.c.m.s. corresponding to a molecular formula of $C_{21}H_{26}O_6$. The e.i. mass spectrum and ^1H n.m.r. spectral data matched those of caulerpenyne (Appendix 1) a toxic secondary metabolite first isolated from *C. prolifera*. Caulerpenyne **(4)** and related secondary metabolites will be discussed in more detail in Chapter 6.

The most polar band obtained by p.t.l.c. contained a compound which was brown-grey in colour, with a small amount giving intense coloration. The ^1H n.m.r. spectrum of **(16)** (Figure 3.33) indicated the presence of functional groups containing oxygen or nitrogen. The u.v.-visible spectrum of this compound (Appendix 1) indicated that **(16)** was a pigment with l.c.-m.s. indicating a molecular weight of 870 a.m.u. Comparison of the spectral data of **(16)** with the literature available on pigments resulted in the identification of **(16)** as pheophytin a; a compound differing from chlorophyll *a* by the lack of a central magnesium atom and two hydrogen atoms. Pheophytin a was present in all of the *Caulerpa* species investigated for this thesis and served as a useful marker for delineating the region in which terpenoid secondary metabolites were likely to be located.

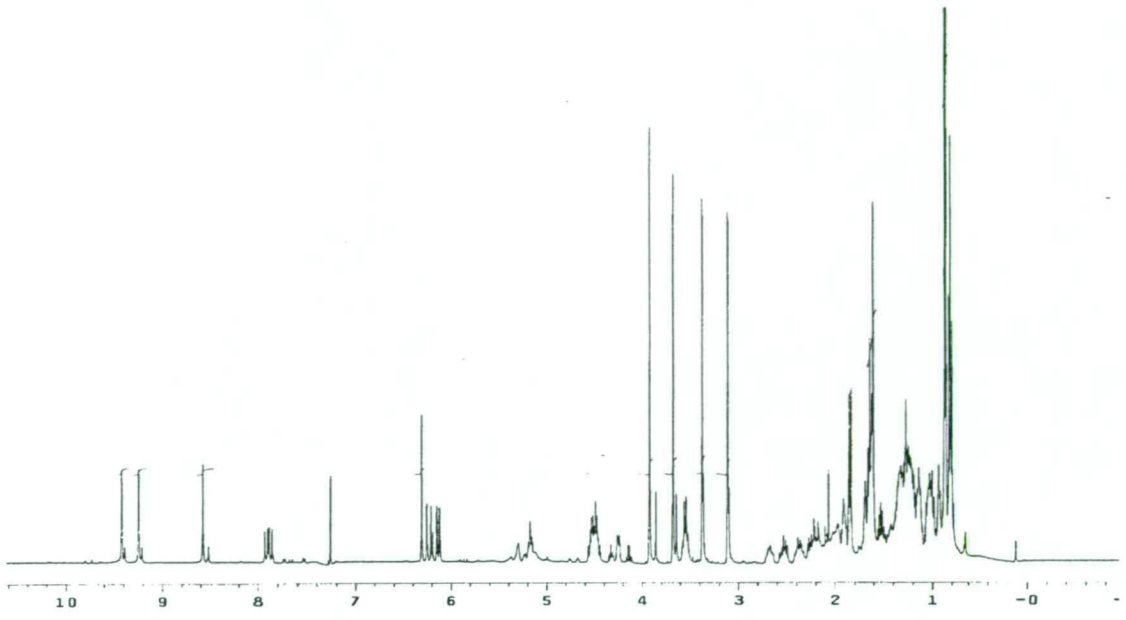
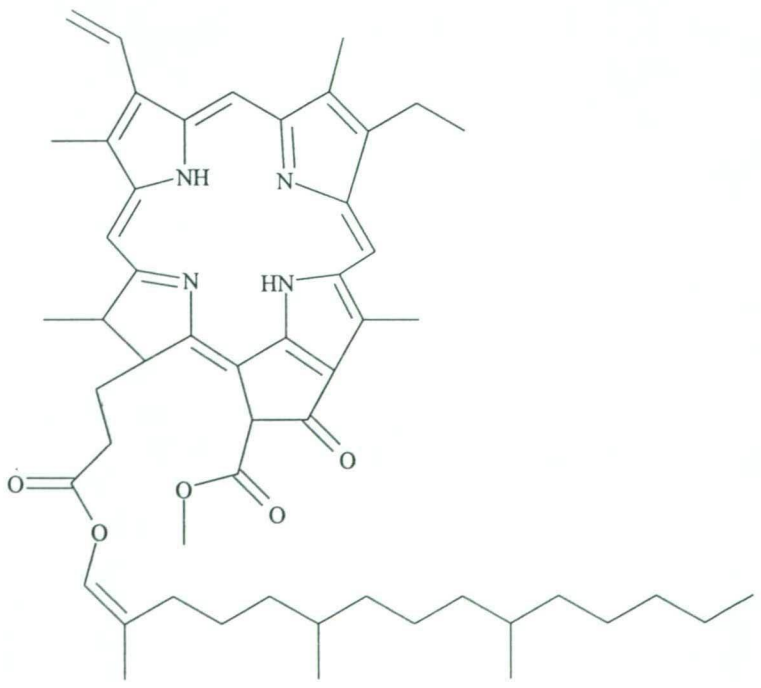


Figure 3.33 ^{1}H n.m.r. of (16) (400MHz, $CDCl_3$)



(16)

3.10 Geographic variation of *C. trifaria* metabolites

The different collections of *C. trifaria* investigated for this thesis showed considerable variation in the relative proportions of secondary metabolites present. The crude extracts often contained an unstable dialdehyde compound containing ^{1}H n.m.r. signals at 9.7 and 9.8 ppm **(17)** (despite intensive efforts this compound was unable to be isolated), compound **(8)**, the acetoxybutadiene group of compounds **(1)-(5)** and other compounds that were unable to be isolated due to either instability and/or being present in small amounts. Figure 3.34 illustrates the geographic variation of some of the secondary metabolites present in five of the *C. trifaria* collections.

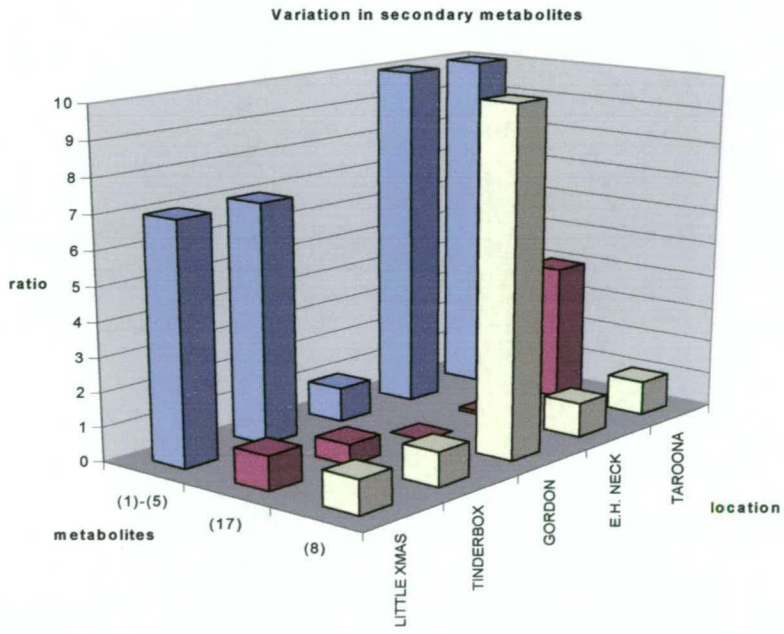


Figure 3.34 Geographic variation of *C. trifaria* secondary metabolites

The metabolite **(5)** was the major secondary metabolite present in most of the collections with there being an inverse relationship between **(5)** and metabolite **(8)** when this was not the case (Gordon collection). No other geographic patterns could be discerned between the various collections of *C. trifaria* undertaken for this thesis.

3.11 Instability of secondary metabolites

The secondary metabolites present in the crude extract of *C. trifaria* also showed instability when subjected to temperature changes. To illustrate this instability an extract from Eaglehawk Neck was subjected to the following events:

1. An initial n.m.r. experiment 2 hours after removal from –20 ^{0}C freezer;
2. The extract (in a round bottomed flask) was then immersed in hot water (60^{0}C) for 1 hour and left on a laboratory bench for 48 hours;
3. The flask containing extract was then immersed in boiling water for 30 minutes.

N.m.r. experiments were conducted after each stage and the fluctuations in secondary metabolite concentration are shown in the figure below.

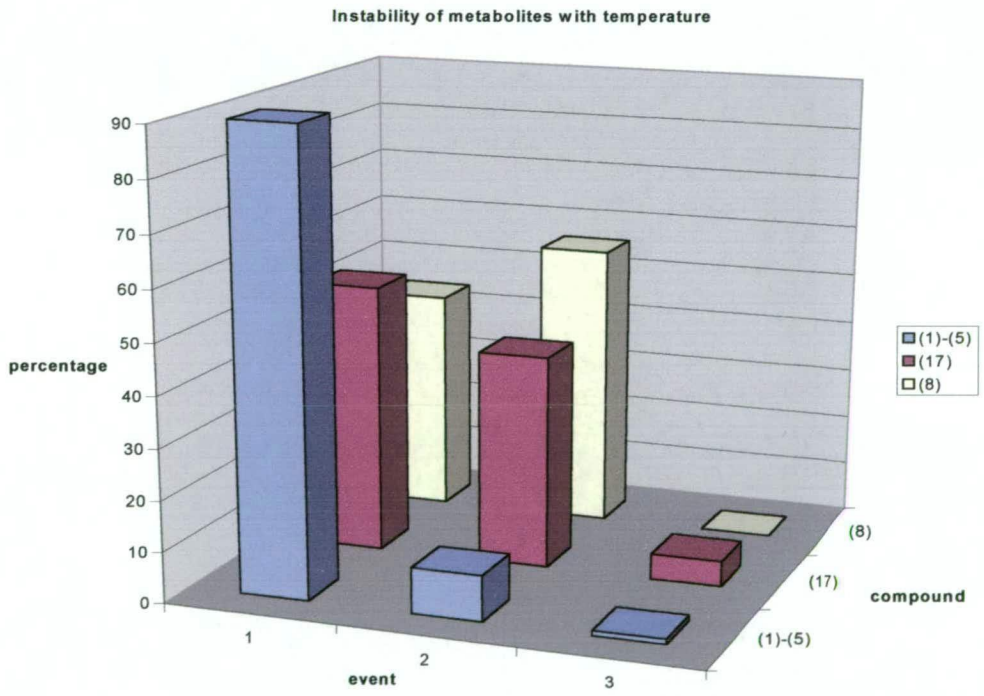


Figure 3.35 Thermal instability of *C. trifaria* secondary metabolites

It can be seen from the results of this experiment that the metabolites were sensitive to temperature changes.

The instability of metabolites was further demonstrated by the general observation that the longer the extracts were kept in storage (even at –20 ^{0}C) the lower the concentration of secondary metabolites became. Generally the compounds that first disappeared on storage were the aldehydic compounds followed by the acetoxybutadiene compounds. After several months at -20^{0}C little evidence of these secondary metabolites remained. This instabilty resulted in eight different collections of *C. trifaria* being made in order to pursue metabolites present in different fractions.

The thermal instability of compound **(8)** was also demonstrated on g.c.-m.s. (Figure 3.36) where despite the n.m.r. and l.c.-m.s. of the compound showing high purity the g.c.-m.s. showed a complex mixture of isomers. This instability of a 1,4 α,β-unsaturated dialdehyde on g.c.-m.s. was to be repeated with similar metabolites isolated from other *Caulerpa* species investigated for this thesis.

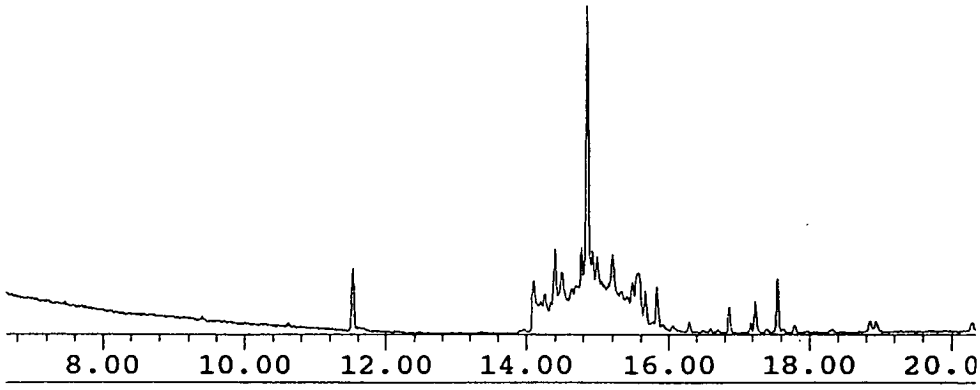


Figure 3.36 Thermal instability of *C. trifaria* secondary metabolite on g.c.

The instability of the secondary metabolites in *C. trifaria* was also responsible for the partial decomposition of some metabolites during purification of extracts. Whilst extracts were monitored by n.m.r. and g.c.-m.s. to ensure that only metabolites originally present in the alga were isolated, it became apparent that some decomposition was occurring during p.t.l.c. and h.p.l.c. This was possibly due to the acidic nature of silica resulting in the production of acetic acid from some of the acetoxybutadiene moieties and acid catalysed decomposition of some of the aldehyde moieties present in the metabolites. However since extensive trialling of other methods of purification resulted in much poorer separation and as supply of *C. trifaria* was plentiful, the methods of silica p.t.l.c. and h.p.l.c. (using both normal and reverse phase) were employed. This partial decomposition explains why the novel dialdehyde compound **(8)** isolated from *C. trifaria,* which in the extract from Gordon appeared to be the major secondary metabolite present before purification, was isolated in lesser amounts than the novel acetoxybutadiene compound **(5)**.

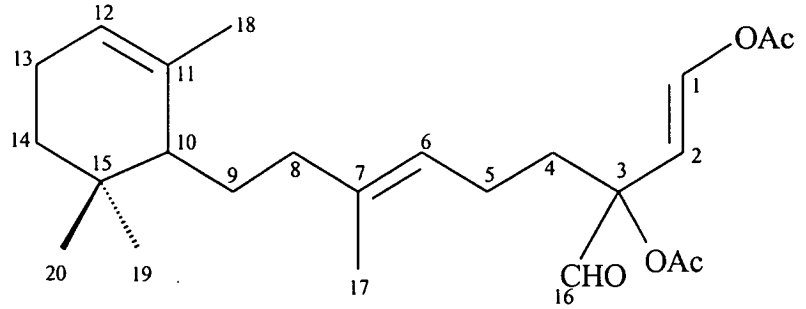
Decomposition of metabolites on silica p.t.l.c. was not as noticeable in other algae studied for this thesis; where again careful monitoring by n.m.r. and g.c.-m.s. was employed to ensure that only metabolites originally present in the algae were isolated. H.p.l.c. methods employed with subsequent algae using acetonitrile and water mixtures on a reverse phase column gave good separation with metabolites containing acetoxybutadiene and aldehyde metabolites and better recovery; suggesting that further investigations of *C. trifaria* should employ such mixtures where appropriate.

3.12 Function of *C. trifaria* metabolites as chemical defense agents

Studies by Paul, Littler and Fenical[13,14] into *Caulerpa* species have demonstrated strong avoidance of *C. ashmeadii* by reef herbivores. This correlated with the presence of biologically active compounds containing acetoxy or aldehyde functional groups in *C. ashmeadii*. The aldehydes showed particular potency in the antimicrobial and ichthyotoxic assays conducted. These studies also found chemical variation, both qualitative and quantitative in different populations of the same species; algae growing in the areas having the highest herbivory produced the greatest concentrations and variations in secondary metabolites.[11] It is probable that the secondary metabolites **(1)-(5)**, **(8)**, **(11)** and **(12)** which contain aldehyde or acetoxy moieties function as chemical defense agents in *C. trifaria* and that the variation and concentration of secondary metabolites is due to similar environmental factors that were found in the studies of *C. ashmeadii*.

An inspection of this spectrum indicates that this p.t.l.c. fraction consists chiefly of a major metabolite with diagnostic peaks at 9.39 , 7.43 (d *J* 12.4 Hz) and 5.42 (d *J* 12.4 Hz) ppm and a minor metabolite with diagnostic peaks at 9.79 and 9.72 ppm. It is also apparent from this spectrum that these metabolites possess signals in the region below 2.4 ppm typical of the substituted dimethylcyclohexene moiety found in the novel secondary metabolites **(5)**, **(8)**, **(11)** and **(12)**.

A review of the spectral data for this fraction after investigations into *C. brownii* (unbranched) (Chapter 4) and *C. flexilis* (Chapter 5) indicate that the major metabolite present possesses ^{1}H n.m.r. spectral data almost identical to that of the novel metabolite **(18)** isolated from *C. brownii* (unbranched) below:



(18)

With the knowledge of separation techniques gained during the investigations of *C. brownii* (unbranched) and *C. flexilis*, isolation of the pure metabolites in this fraction by h.p.l.c. should be readily achievable. The elucidation of the structure of metabolite **(18)** isolated from *C. brownii* (unbranched) is detailed in Chapter 4. It is interesting to note *C. trifaria* and *C. brownii* (unbranched) were found to possess several secondary metabolites in common. This will be discussed further in Chapter 4.

The ^{1}H n.m.r. spectrum of the remaining p.t.l.c. fraction is shown in Figure 3.38.

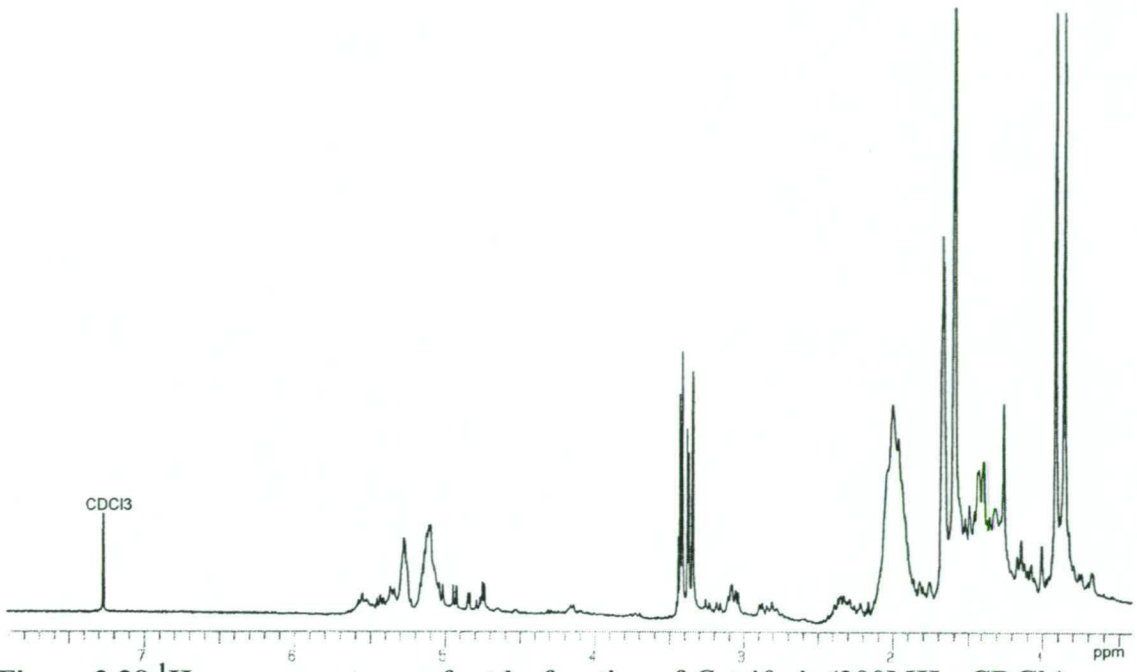


Figure 3.38 ^{1}H n.m.r. spectrum of p.t.l.c fraction of *C. trifaria* (200MHz, $CDCl_3$)

It can be seen from Figure 3.38 that the metabolite(s) contained in this fraction also possess signals consistent with the presence of a substituted dimethyl cyclohexene moiety in the region below 2.1 ppm and interesting signals in the region 3.4 to 3.6 ppm. Signals due to acetoxy moieties in the region 2 to 2.16 ppm are however absent in this spectrum. A reinvestigation of this fraction of *C. trifaria* using the h.p.l.c. techniques developed during subsequent investigations into *Caulerpa* species should lead to the purification and identification of the metabolite(s) present in this fraction of *C. trifaria*.

3.14 References

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Chapter Four

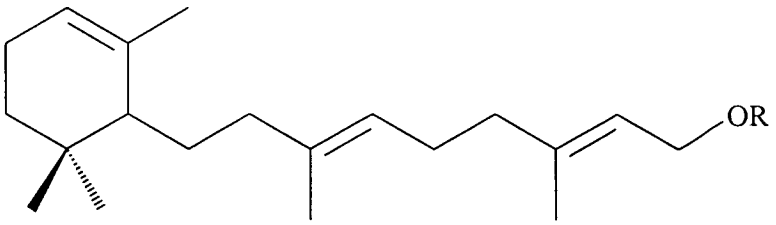
Studies of the Tasmanian green alga

Caulerpa brownii

Contents

4.1 Introduction

This chapter describes separate investigations into the unbranched and branched specimens of *Caulerpa brownii*. The known metabolites **(1)**, **(3)**, **(4)**, **(5)** and **(8)** and the novel metabolites **(6)**, **(7)**, **(11)**, **(15)**, **(16)**, **(19)** and **(20)** were isolated from the unbranched specimens. Metabolite **(8)** was isolated for the first time as a natural product, whilst the novel metabolites **(6)** and **(7)** were previously found in *C. trifaria* (Chapter 3). Metabolite **(1)** was found in one collection of unbranched specimens as a minor component however was the major metabolite present in the branched specimens. The known acetate **(2)** and the novel terpenoid esters **(23)** were also isolated from the branched specimens of *C. brownii*.



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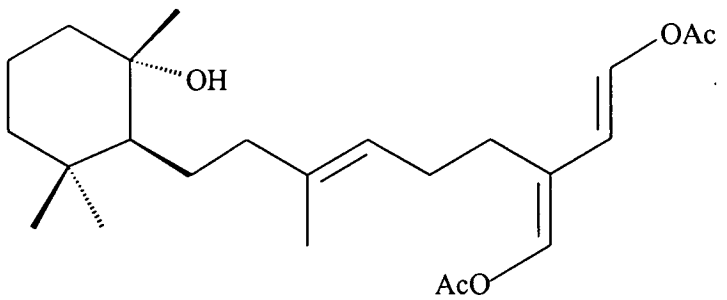

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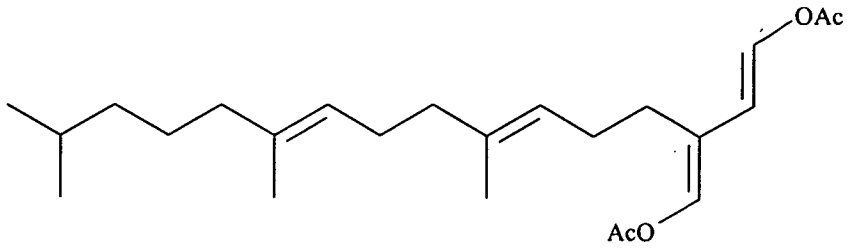

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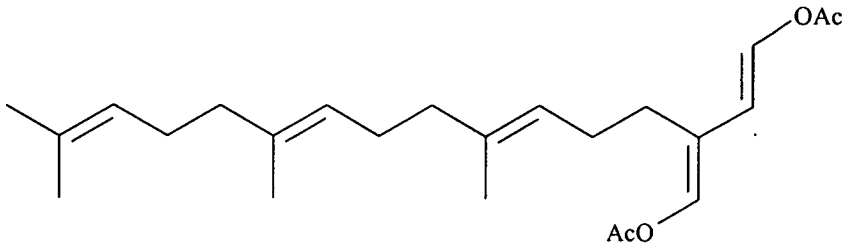

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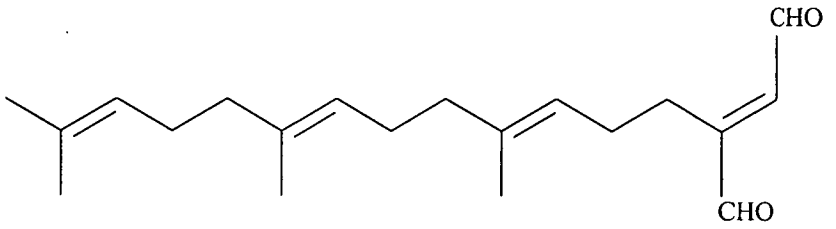

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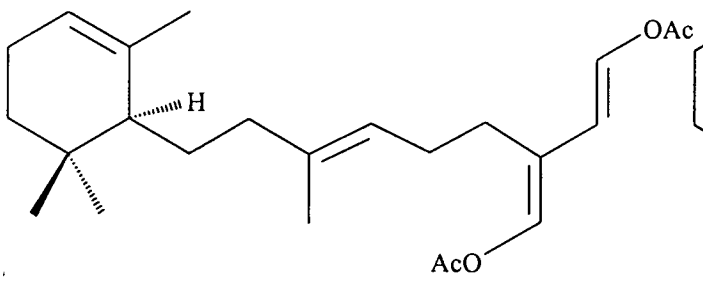

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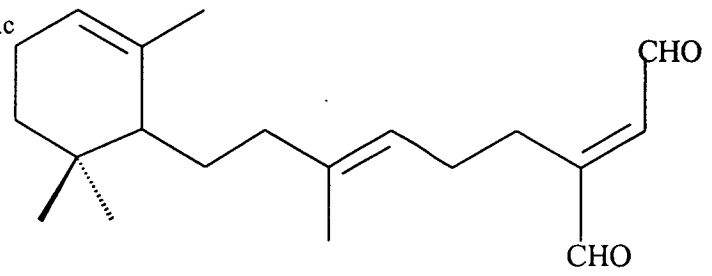

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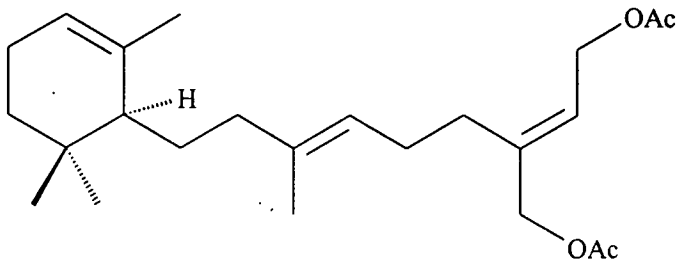


(11)

C. brownii is found along the coastline of Australia from Perth, Western Australia to Walkerville, Victoria and around Tasmania, New Zealand, Chatham Snares and Lord Howe Islands. This green alga is epilithic; commonly being found on rocks and just below low tide level. *C. brownii* often forms a monospecific community and has been found down to depths of 42 metres.[1]

C. brownii possesses a robust stolon 3-5 mm in diameter which is densely covered with simple ramuli 1-2.5 mm long and 300-400 μm in diameter tapering abruptly to a spinous tip. The fronds are erect and colored medium to dark green with either a simple or several irregularly branched axes. During our collections of *C. brownii* it was apparent that those specimens with a simple axis (Figure 4.1A) grew separately from those specimens possessing several axes (Figure 4.1B), although both types were often found in the same general area.

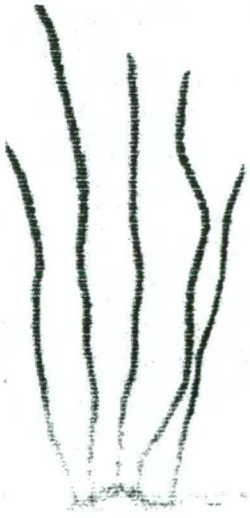


Figure 4.1A (unbranched)

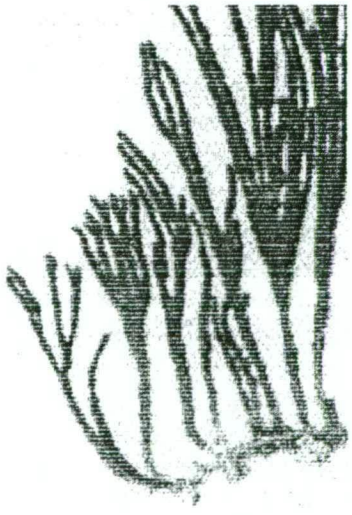


Figure 4.1B (branched)

For this thesis, collections of *C. brownii* were divided into branched and unbranched specimens and investigated separately. The results of these investigations revealed consistently that the secondary metabolites present in the unbranched specimens were different from those found in the branched specimens. It may be that this evidence supports the reclassification of branched and unbranched specimens as sub or separate species of *C. brownii.* Figure 4.2 shows *C. brownii* (unbranched) growing amongst other *Caulerpa* species from one of our collection sites.

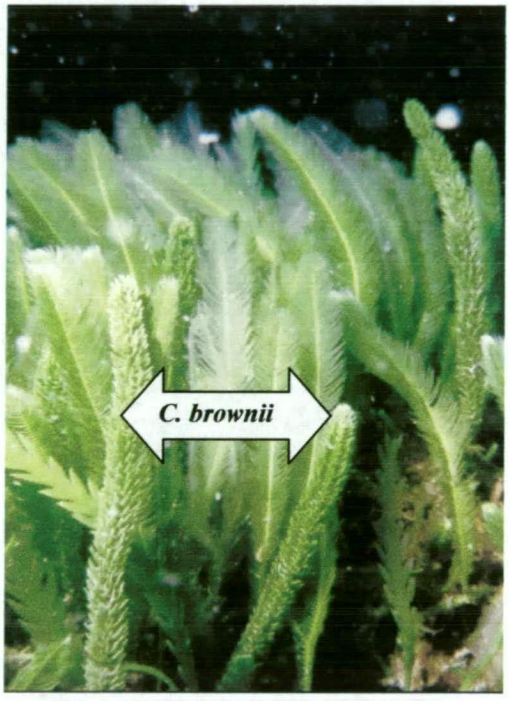
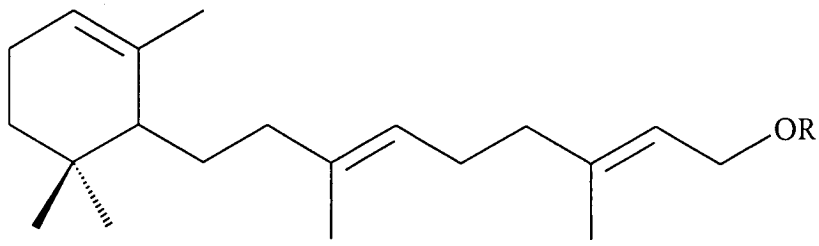


Figure 4.2 *C. brownii* (unbranched)

4.2 Previous investigations of *C. brownii*

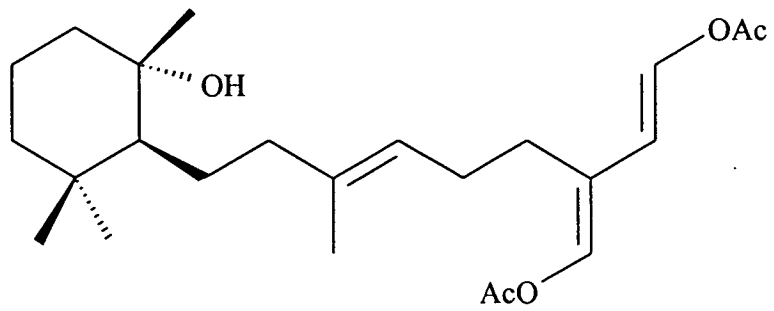
In 1976 the Blackman research group investigated *C. brownii* from Cook's Corner, (Freycinet Peninsula, East Coast of Tasmania) for the presence of secondary metabolites. Freshly collected *C. brownii* was exhaustively extracted at 0^0C with chloroform-methanol (1:1 v/v) and the concentrated extract partitioned between hexane and aqueous methanol. The hexane fraction was purified by dry column chromatography on silica gel using hexane-chloroform as the eluent; yielding the novel diterpene alcohol caulerpol **(1)** (9.5 % dry weight) and its acetate **(2)**.[2]



(1) R = H **(2) R = Ac**

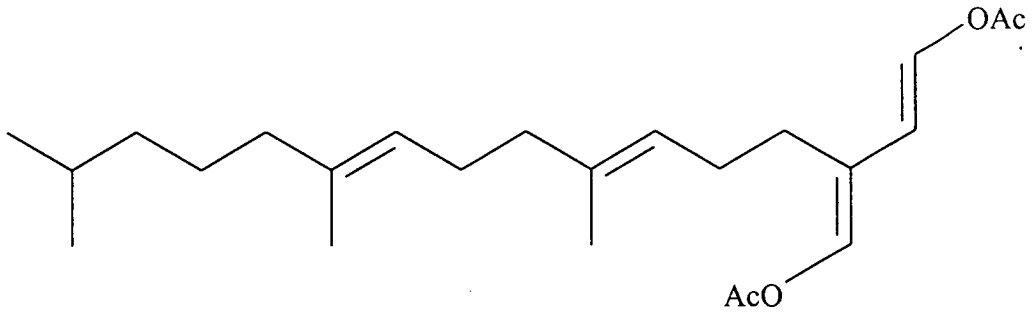
In 1983 Capon, Ghisalberti and Jefferies[3] investigated five *Caulerpa* species from Western Australia for the presence of terpenoid secondary metabolites, including *C. brownii*. Specimens of *C. brownii* collected along the coast of Western Australia between Augusta and Lancelin were homogenised and extracted with DCM:MeOH (1:1). The DCM fractions revealed the presence of trace amounts of compounds which were unable to be isolated by p.t.l.c.[3]

In 1985, Paul and Fenical reported the isolated of a novel secondary metabolite (3) from a collection of *C. brownii* from Flinders Reef near Melbourne, Victoria.[4]

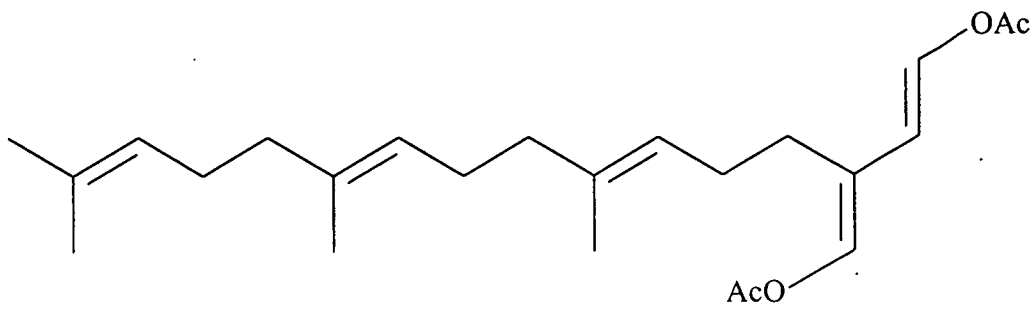


(3)

The alcohol **(3)** was isolated as 20 % of the organic extract after silica gel h.p.l.c. (EtOAc:iso-octane 7:13). The known diterpenoids trifarin **(4)** and didehydrotrifarin **(5)** were also isolated from the organic extract (5 % combined). Compound **(3)** showed antibacterial activity towards the human pathogenic bacteria *Staphylococcus aureus* and *Bacillus subtilis* and inhibitory activity towards the marine bacteria *Vibrio harveyi* and *V. leiognathi.* Compound **(3)** was also active against *E. coli* and *V. anguillarum.*[4]



(4)



(5)

4.3 Collection, extraction and isolation

C. brownii was collected from Spring Beach on the East Coast of Tasmania in March and May of 1999 and November and July of 2000 (Chapter 1, Figure 1.6B). The branched and unbranched varieties of *C. brownii* were observed growing in separate patches in the same general area at Spring Beach.

Upon return to the laboratory each collection was sorted into branched and unbranched specimens and then freeze-dried and extracted without delay. The petroleum ether extracts were then screened using ^{1}H n.m.r.spectroscopy, t.l.c. and g.c.-m.s. analysis. The g.c. profiles of the unbranched and branched specimens are shown in Figure 4.3 below, in mirror image format. The upper image is the profile of the unbranched specimens. It can be seen that the two g.c. profiles are quite different.

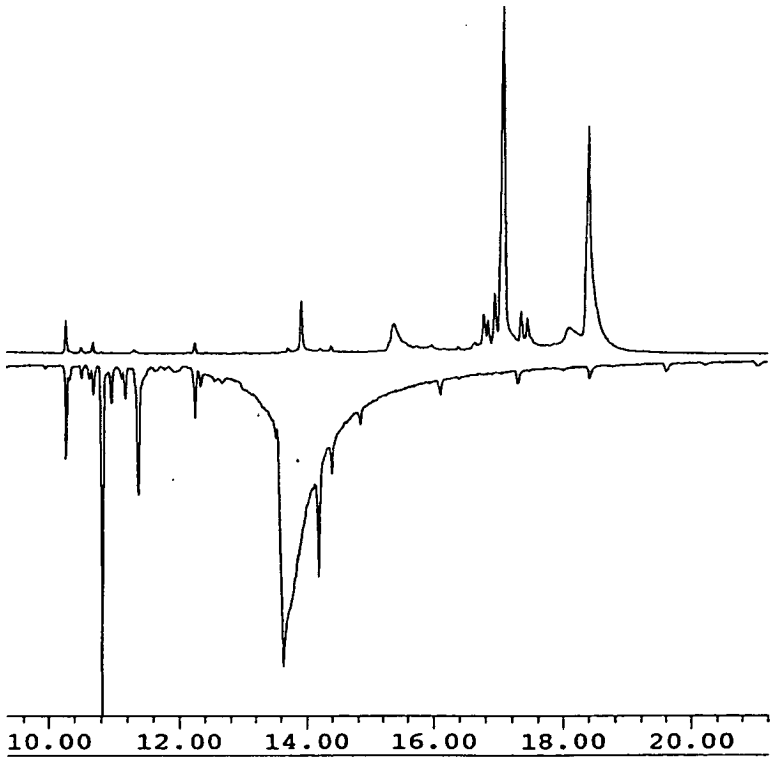


Figure 4.3 G.c. profiles of *C. brownii* extracts

In contrast Figure 4.4 shows the g.c. profile of the unbranched *C. brownii* in mirror image format against the g.c. profile for *C. trifaria*. The *C. brownii* (unbranched) is the lower image in this figure. The m.s. data for these two g.c. profiles suggested that some metabolites possessing similar structures were present in both *Caulerpa* species.

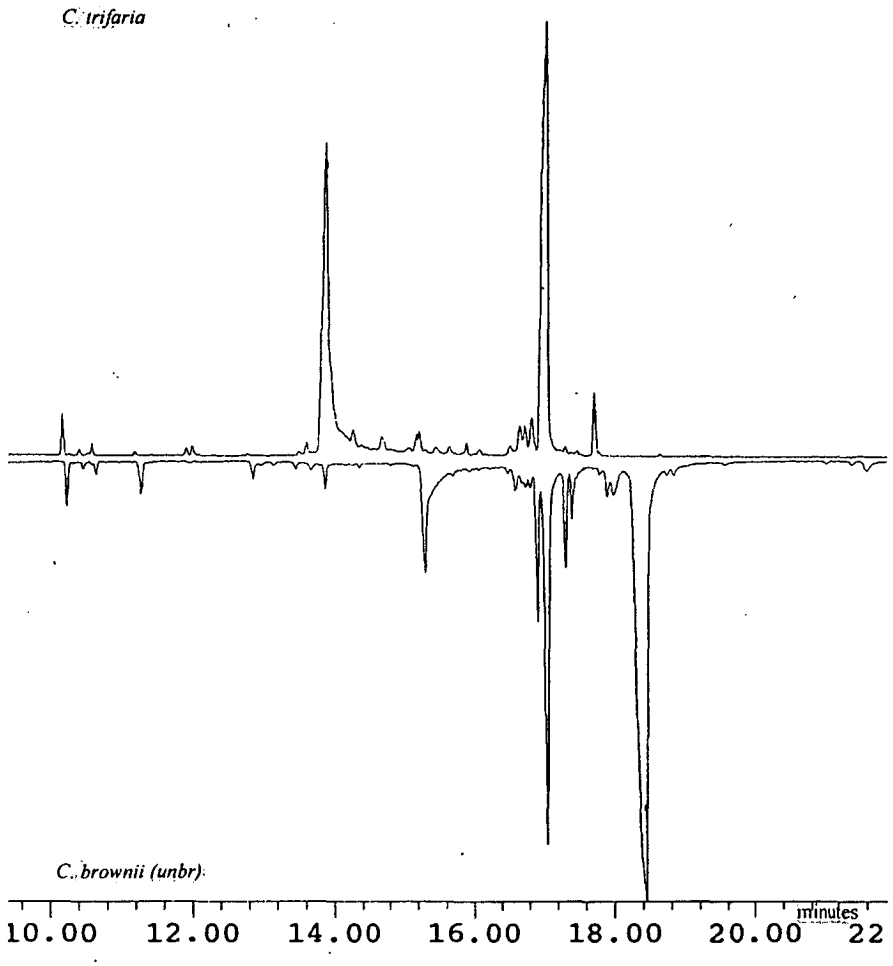


Figure 4.4 G.c. profiles of *C. trifaria* (upper) and *C. brownii* (unbranched) (lower) extracts

4.4 *C. brownii* (unbranched)

The ^{1}H n.m.r. spectrum of the extract of the unbranched collection (Figure 4.5) showed signals in the 7.5 to 5.8 ppm region of the spectrum, suggestive of secondary metabolites containing an acetoxybutadiene moiety. Smaller signals above 9.0 ppm indicated the presence of compounds containing aldehydic moieties while some extracts showed smaller signals in the region between 4.4 and 4.7 ppm suggestive of allylic acetoxy groups.

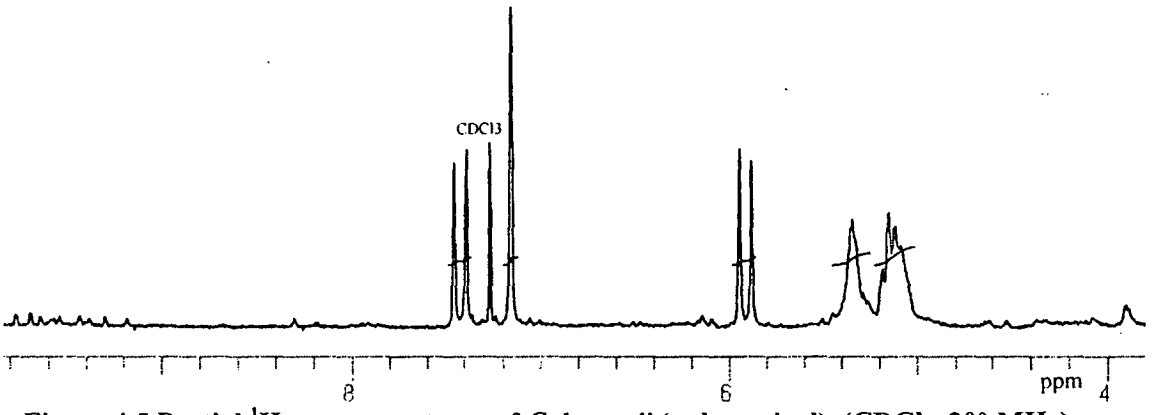


Figure 4.5 Partial ^{1}H n.m.r. spectrum of *C. brownii* (unbranched), ($CDCl_3$, 200 MHz)

The presence of signals at 0.85 and 0.92 ppm in the ^{1}H n.m.r. spectrum of the extract suggested that some of these secondary metabolites contained a monocyclic moiety with a geminal dimethyl substituent.

The presence of terpenoid secondary metabolites was further supported by the g.c.-m.s. profile (Figure 4.6) which indicated the presence of a number of compounds in the molecular weight range 300-430 a.m.u. with fragmentation patterns suggestive of acetoxy and aldehyde moieties.

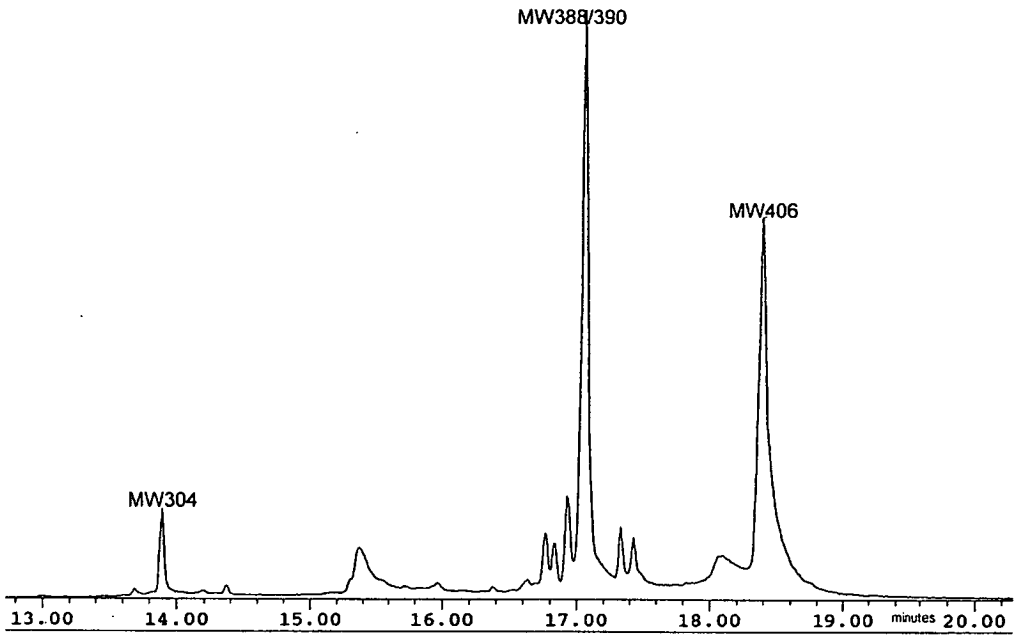


Figure 4.6 G.c. profile of *C. brownii* (unbranched), MW ions obtained from m.s. data of peaks

The t.l.c. of the petroleum ether extract of *C. brownii* revealed a number of ultraviolet active spots in the region above pheophytin a (Figure 4.7).

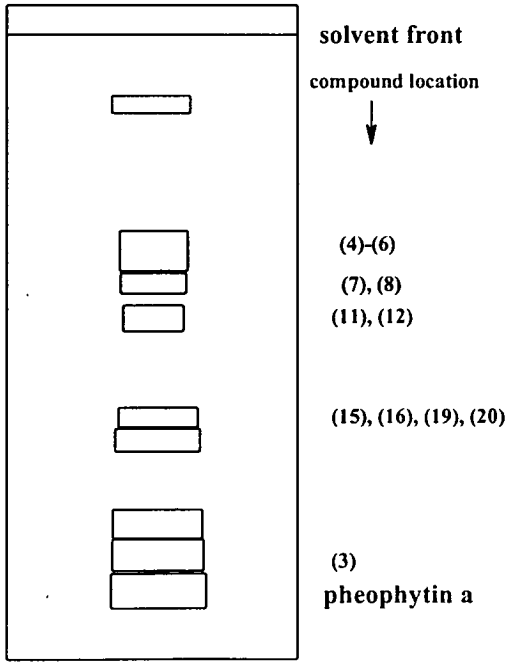
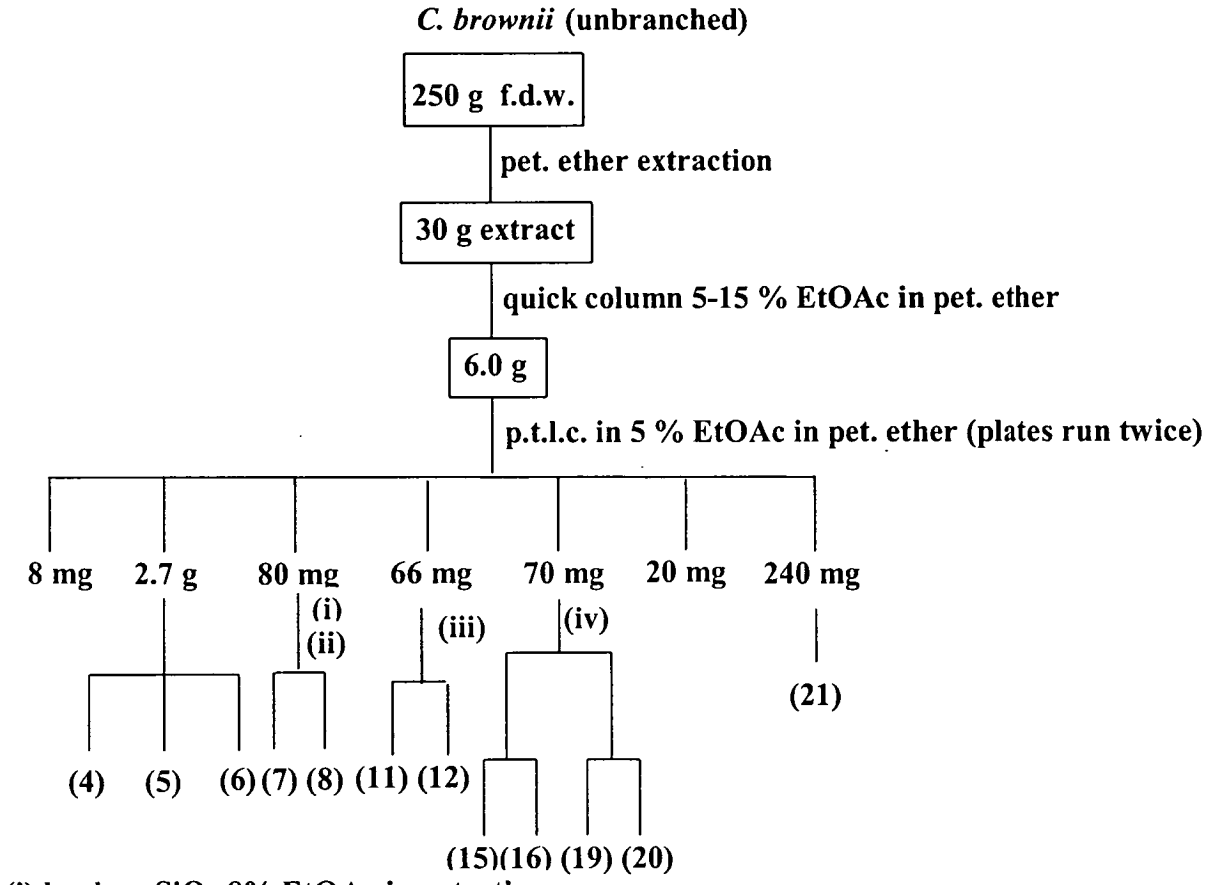


Figure 4.7 T.l.c. of *C. brownii* (unbranched) (5 % EtOAc in pet. ether)

The petroleum ether extract of *C. brownii* (unbranched) was then subjected to the purification steps shown in Scheme 4.1.



Scheme 4.1 Purification sequence for *C. brownii* (unbranched)

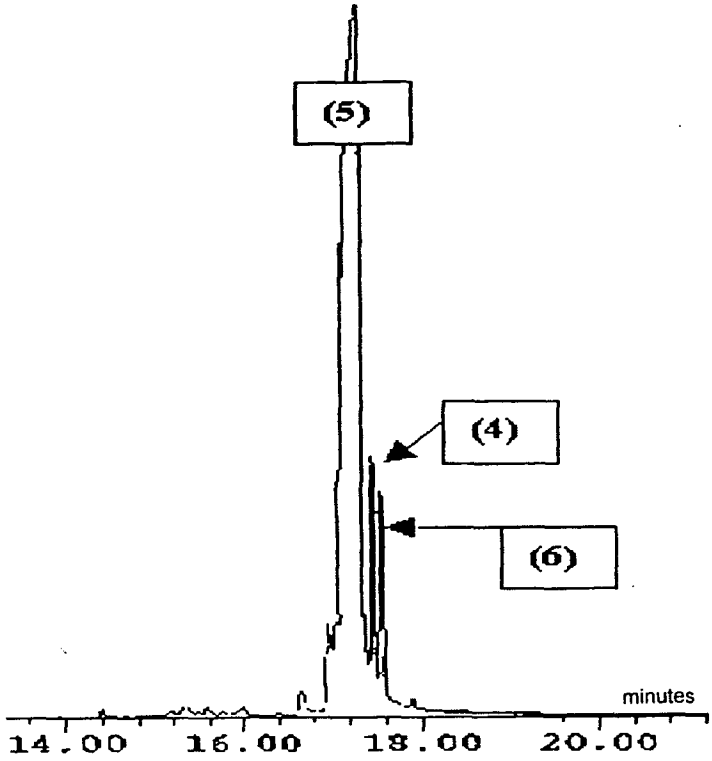
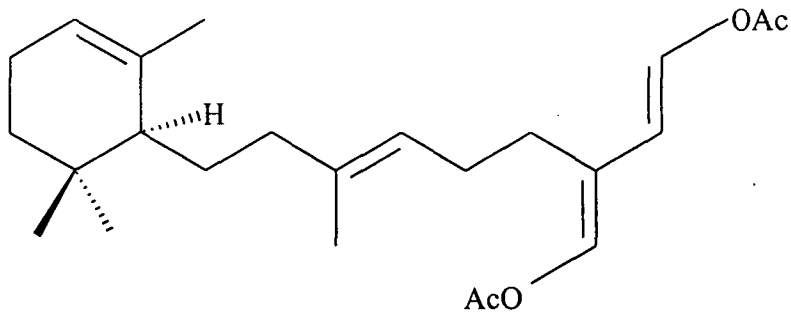


Figure 4.9 G.c. profile of the major fraction of *C. brownii* (unbranched)

The m.s. spectral data indicated that metabolite **(6)** was identical to the novel secondary metabolite identified by us as the major metabolite in *C. trifaria.*



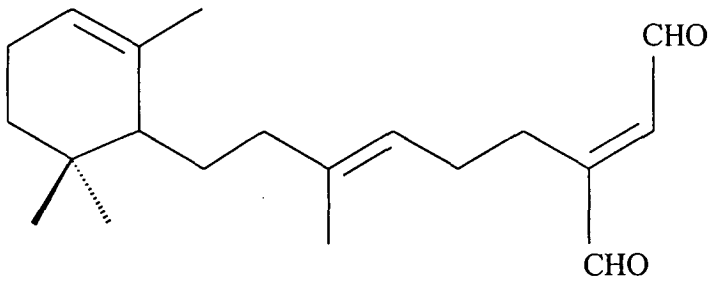
(6)

Since the secondary metabolites in this fraction were all known, no further efforts were made to separate them. The mass spectral data for the metabolites **(4)**, **(5)** and **(6)** are contained in Appendix 2 (Figure 2.1-2.3).

4.6 Structure elucidation of metabolites (7) and (8)

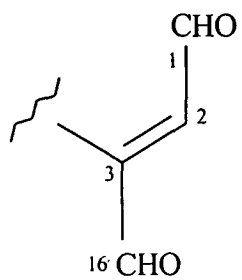
The next most polar band obtained by p.t.l.c. (5 % EtOAc in pet. ether) was purified by normal phase h.p.l.c. (SiO_2, 8 % EtOAc in pet. ether) to give a mixture of two compounds, in a 70:30 ratio, both with molecular weight 302. Subsequent h.p.l.c. analysis (C 18, 5 % H_2O in acetonitrile) resulted in the successful separation of these isomers.

The ^1H n.m.r spectrum (Appendix 2, Figure 2.4) and the l.c.-m.s. data for the minor component (7) confirmed that (7) was identical to the novel 1,4-α,β-unsaturated dialdehyde compound isolated from *C. trifaria* (Chapter 3).



(7)

The ^1H n.m.r. spectrum (Figure 4.10) of the major component of this fraction was identical above 6.50 ppm to the corresponding region in the ^1H n.m.r. spectrum of (7); indicating that an identical 1,4-α,β-unsaturated dialdehyde moiety was present in (8).



The remainder of the ^{1}H n.m.r. spectrum closely resembled the corresponding region of the ^{1}H n.m.r. spectrum of didehydrotrifarin, containing signals for methyl groups at 1.52, 1.58, 1.59 and 1.67 ppm and signals due to methylene protons in the region 1.90 – 2.30 ppm.

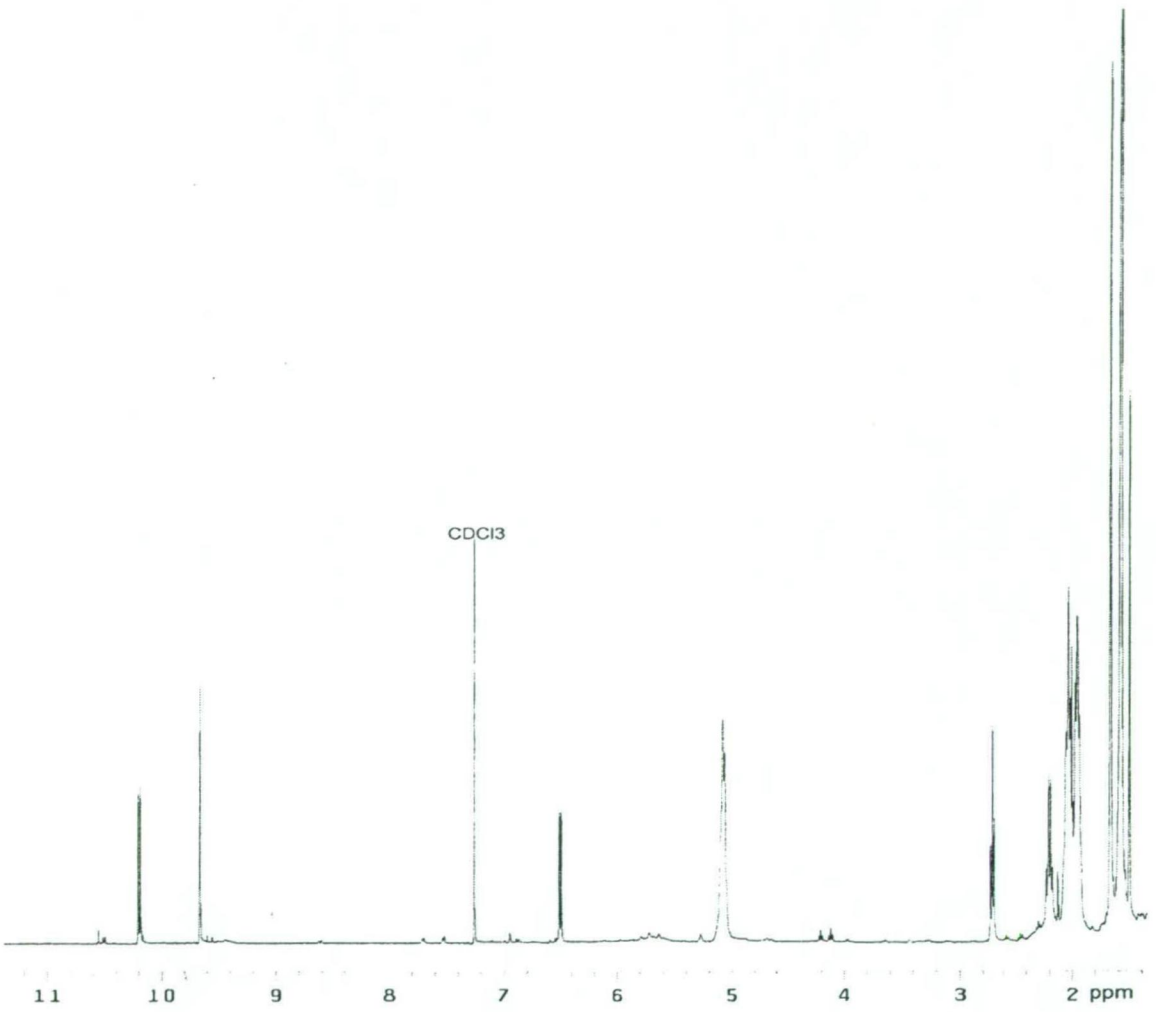


Figure 4.10 ^{1}H n.m.r. spectrum of (8) (400MHz, $CDCl_3$)

Furthermore the ^{13}C n.m.r. spectrum of **(8)** (Figure 4.11) included signals for C 5 to C 20 of **(8)** that were identical to the corresponding signals in didehyrotrifarin **(5)** (Appendix 1, Figure 1.7). The DEPT spectrum for **(8)** also gave multiplicities identical to the C 5 to C 20 moiety of didehyrotrifarin (**5**).

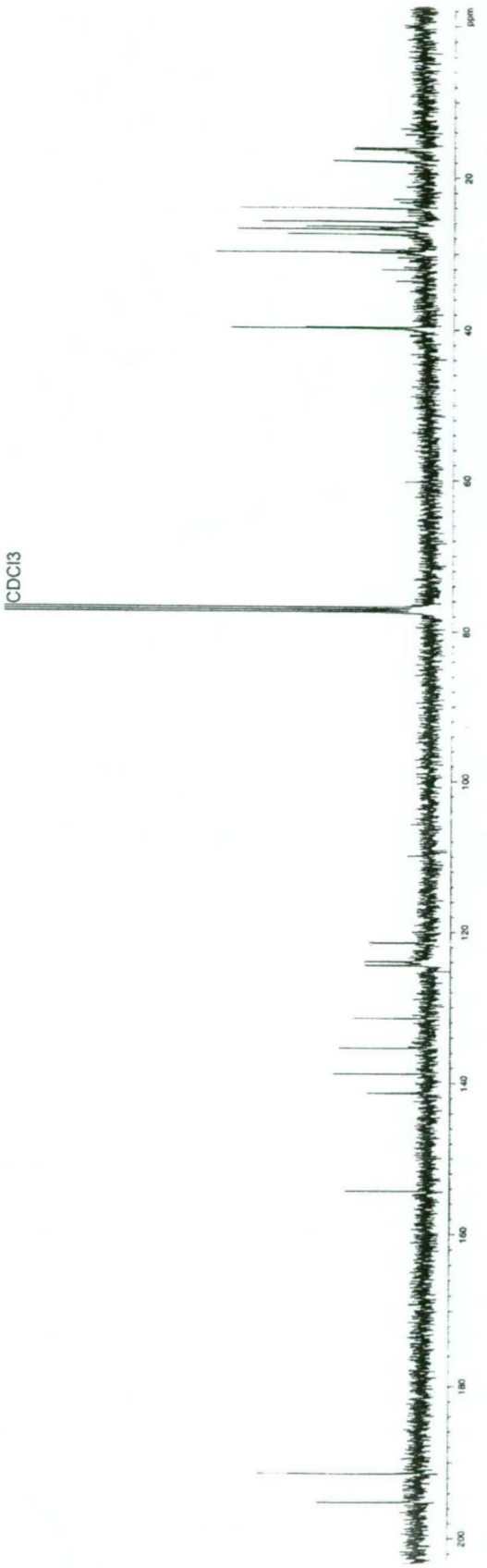
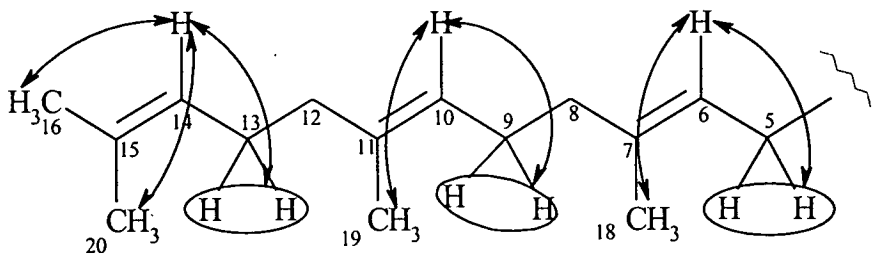
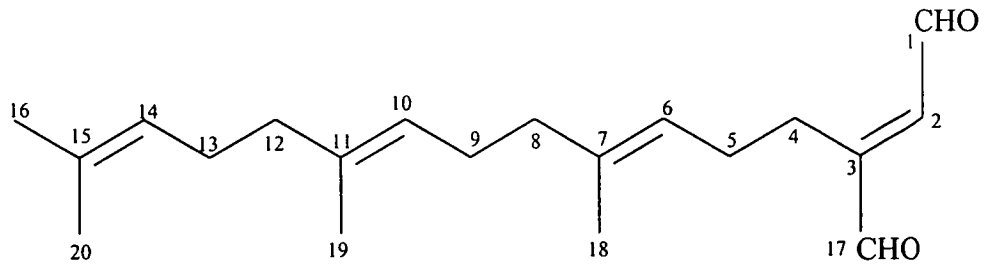


Figure 4.11 ^{13}C n.m.r. spectrum of (8) (400 MHz, $CDCl_3$)

A 2 D COSY n.m.r. experiment (Figure 4.12) showed connections between the H 6 methine proton at 5.08 ppm and the H 5 methylene protons at 2.21 ppm and also between the H 10 and H 14 methine protons at 5.08 ppm and the H 9 and H 13 methylene protons at 1.96 ppm and 2.04 ppm. Long range COSY connections (^4J) were also seen between the methyl group proton signals at 1.51-1.67 ppm and the methine protons at 5.08 ppm.



A COSY connection was also seen between the H 4 methylene protons at 2.72 ppm and the H 5 methylene protons at 2.21 ppm thus linking these two substructures together to give the structure of **(8)** below:



(8)

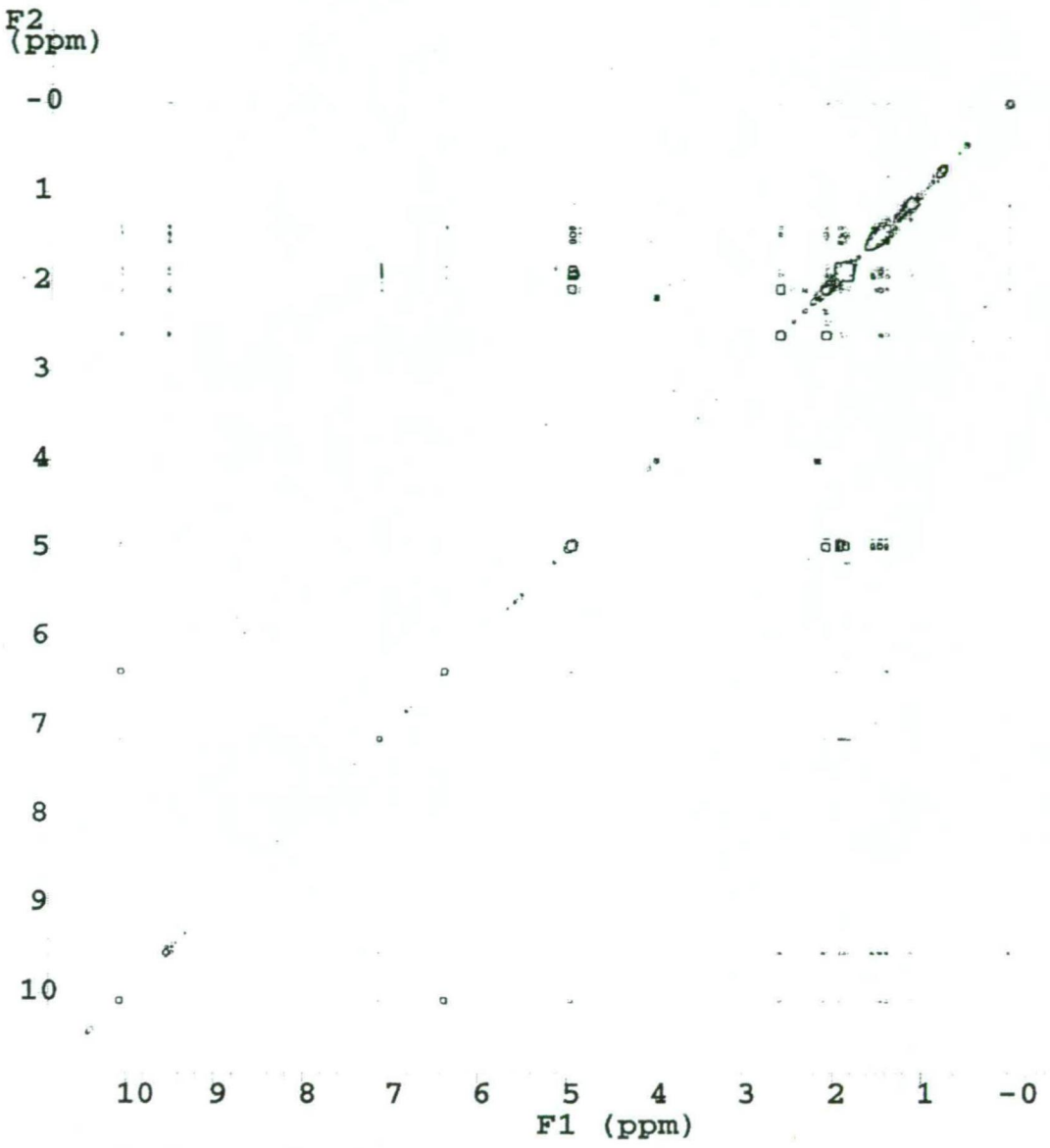
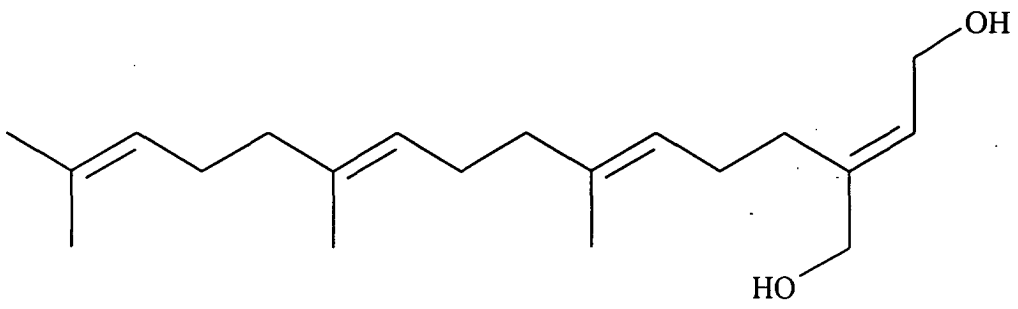


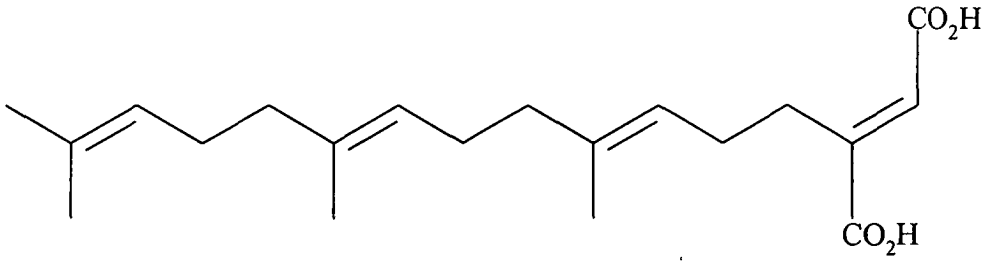
Figure 4.12 COSY n.m.r. spectrum of (8) (100MHz, $CDCl_3$)

Compound **(8)** has been previously synthesised by manganese dioxide oxidation of the diterpene **(9)** first isolated from the species *Kingianthus paradoxus*[5] in 1980.



(9)

More recently in 1996, **(8)** was synthesised as an intermediate in the total synthesis of schizostatin **(10)**, a potent and selective inhibitor of squalene synthesis.[6] Schizostatin **(10)** was previously isolated as a fungal metabolite from the mushroom *Schizophyllum commune* Fr.[7] The relevant ^{1}H n.m.r. data for compound **(8)** obtained from *C. brownii* (unbranched) and the two synthetic products discussed above are shown in Table 4.1 and it can be seen that the ^{1}H n.m.r. values are almost identical. As far as we know our work is the first report of **(8)** as a natural product. 1,4-α,β-unsaturated dialdehydes will be discussed in detail in Chapter 5.



(10)

3.13 Future directions for *C. trifaria* research

C. trifaria was the first seaweed investigated during the course of this thesis. The instability of some of the secondary metabolites resulted in some fractions not being fully investigated before decomposition. A refinement of the techniques learnt during the *C. trifaria* investigation however led to quicker and more successful investigations of subsequent seaweeds. The subsequent availability of more sophisticated n.m.r. and h.p.l.c. techniques also aided subsequent work. Further work on this seaweed is warranted in the future to purify two p.t.l.c. fractions which consistently showed evidence of terpenoid secondary metabolites by ^{1}H n.m.r. and l.c.-m.s. The ^{1}H n.m.r. spectrum of one of these fractions is shown in Figure 3.37.

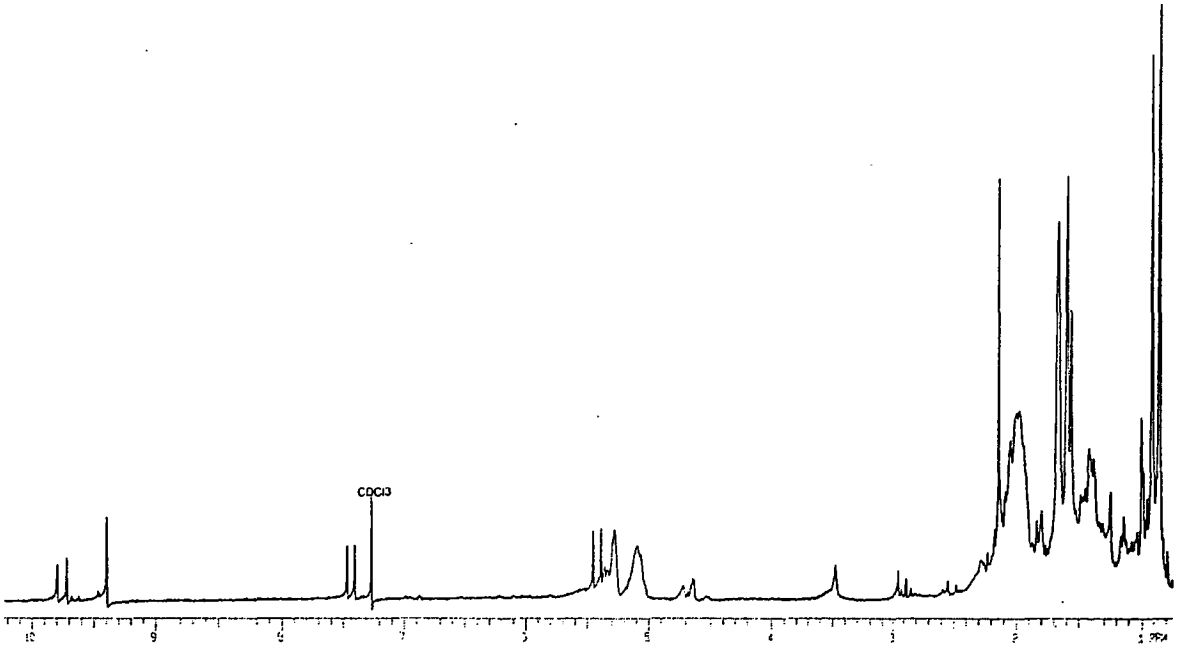


Figure 3.37 ^{1}H n.m.r. spectrum of p.t.l.c. fraction

Hydrogen No.	^{1}H n.m.r. [5] (ppm) (270 MHz, $CDCl_3$)	^{1}H n.m.r. [6](ppm) (400 MHz, $CDCl_3$)	^{1}H n.m.r. (8) (ppm) (400 MHz, $CDCl_3$)
H-1	10.22 (d, J=7.5 Hz)	10.21 (d, J=7.5 Hz)	10.21 (d, J=7.5 Hz)
H-2	6.51 (d, J=7.5 Hz)	6.51 (d, J=7.5 Hz)	6.50 (d, J=7.5 Hz)
H-4	2.73 (br t)	2.73 (t, J=7.5 Hz)	2.72 (t, J=7.5 Hz)
H-5	2.22 (d t)	2.21 (q, J=7.5 Hz)	2.21 (q, J=7.5 Hz)
H-6/10/14	5.10 (3H, br t)	5.06-5.12 (3H, m)	5.09 (3H, br s)
H-8/9/12/13	2.02 (8H, m)	1.95-2.07 (8H, m)	1.94-2.05 (8H, m)
H-16	1.69 (br s)	1.68 (br s)	1.67 (br s)
H-17	9.67 (s)	9.67 (s)	9.67 (s)
H-18, 19, 20	1.61 (6H, s) 1.54 (3H, s)	1.59, 1.60, (6H, s) 1.54 (3H, s)	1.58, 1.59 (6H, s) 1.51 (3H, s)

Table 4.1 ^{1}H n.m.r. spectroscopic data for compound (8)

4.7 Structure elucidation of metabolites (11) and (12)

The next most polar fraction obtained by p.t.l.c. gave the g.c. profile shown in Figure 4.13.

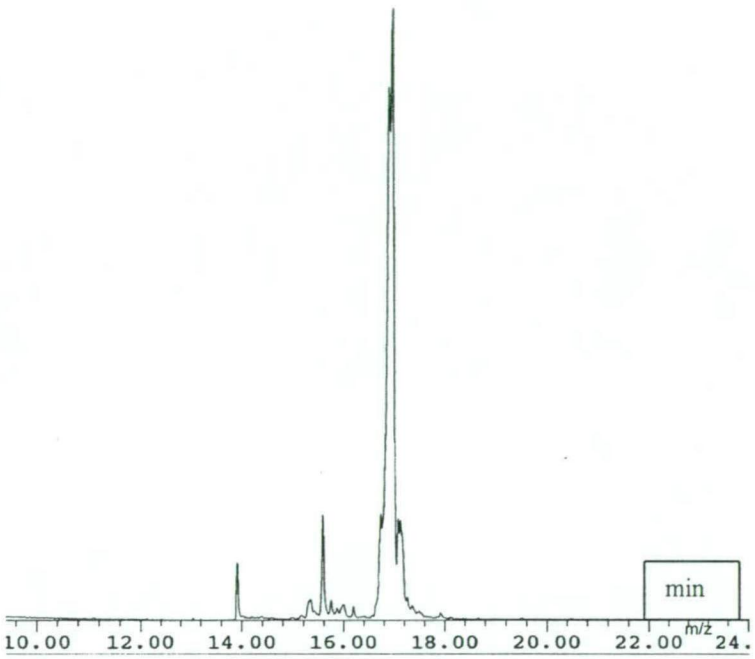


Figure 4.13 G.c. profile of *C. brownii* (unbranched) p.t.l.c. fraction

The mass spectra of the major two peaks in this chromatogram were identical (Figure 4.14) with both showing the highest molecular ion at *m/z* 390 a.m.u. corresponding to a molecular formula of $C_{24}H_{38}O_4$. Peaks at *m/z* 330 and 270 a.m.u. were consistent with the presence of two acetoxy groups (sequential loss of acetic acid = 60 units).

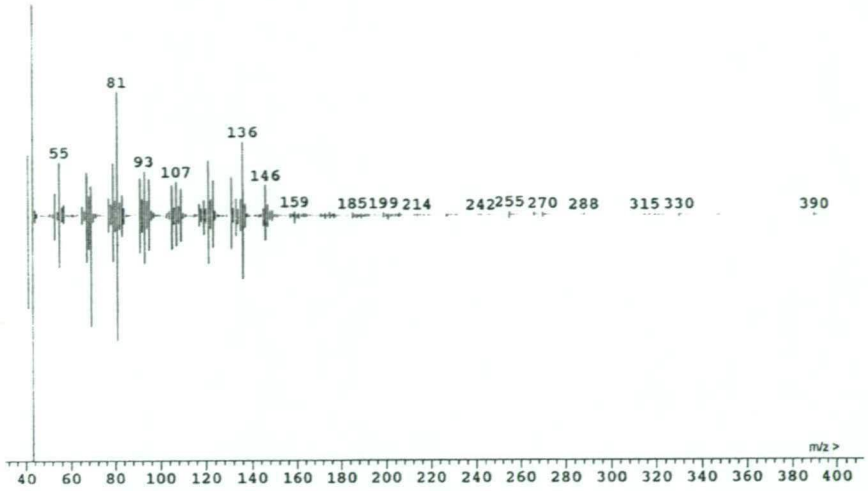


Figure 4.14 Mass spectrum match of two major peaks in Figure 4.13

The ^{1}H n.m.r. spectrum of this fraction (Figure 4.15) contained signals consistent with the presence of a 1,3,3-trimethyl substituted cyclohexene moiety in the region below 2.3 ppm and two sharp singlets at 2.05 and 2.07 ppm typical of acetoxy groups.

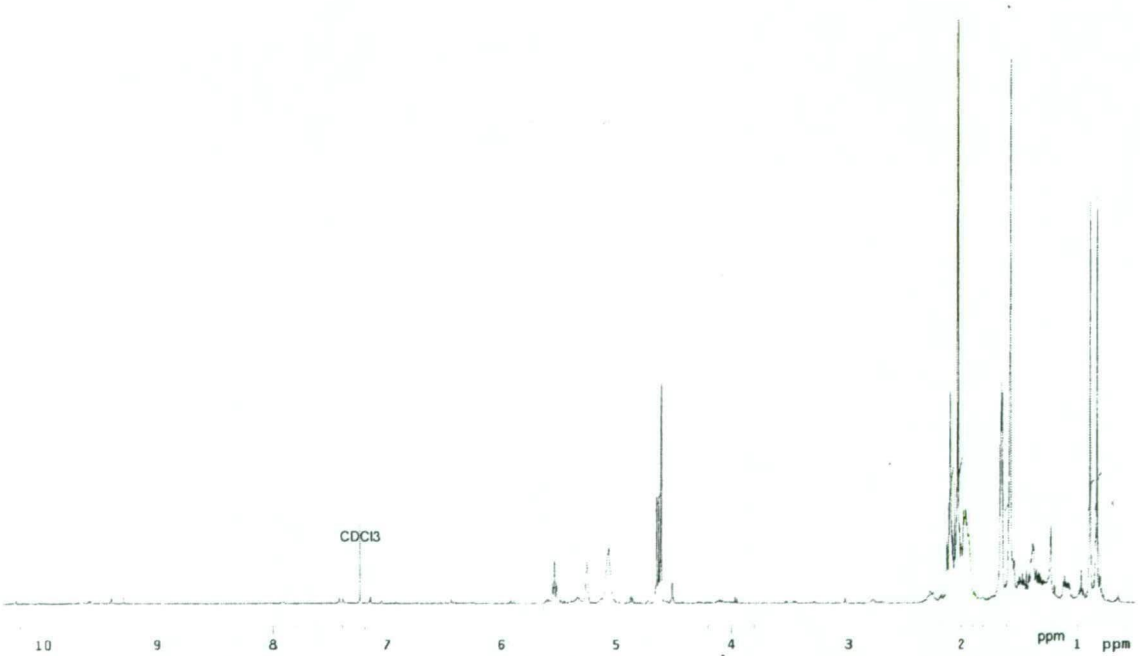


Figure 4.15 ^{1}H n.m.r. spectrum of *C. brownii* (unbranched) p.t.l.c. fraction

This p.t.l.c. fraction was then subjected to reverse phase h.p.l.c. (C18, 5 % EtOAc in MeOH) to give two fractions with almost identical n.m.r. spectral data.

. The ^{1}H n.m.r. spectrum of the slightly less polar metabolite **(11)** (Figure 4.16) included three signals in the region 5.0 to 5.56 ppm characteristic of protons attached to carbon-carbon double bonds and a doublet at 4.66 ppm integrating for two protons. This signal at 4.66 ppm which showed coupling to the proton signal at 5.56 ppm and a singlet at 4.63 ppm, also integrating for two protons indicated the presence of protons attached to oxygenated carbon atoms. The remainder of the ^{1}H n.m.r. spectrum below 2.30 ppm was very similar to the corresponding region of the novel monocyclic metabolite **(6)** obtained from *C. trifaria* suggesting the presence of a 1-methyl 3,3-dimethyl-substituted cyclohexene moiety in the structure of **(11)**.

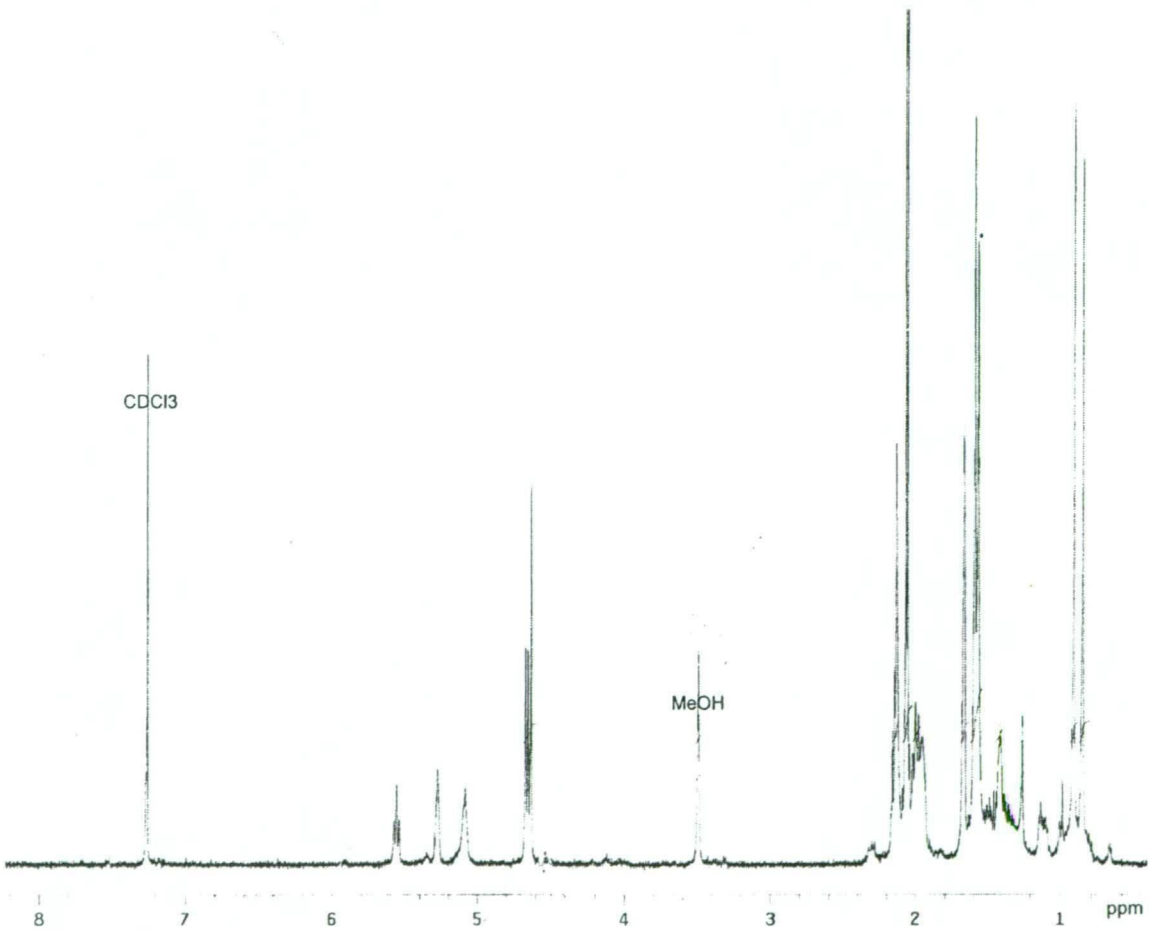


Figure 4.16 ^{1}H n.m.r. spectrum of (11) (400MHz, $CDCl_3$)

The ^{13}C n.m.r. spectrum of **(11)**(Figure 4.17), (Table 4.2) contained twenty four resonances; two signals characteristic of carbonyl carbons at 171.0 and 170.9 ppm, six signals in the vinyl region between 140 and 120 ppm and two signals at 60.6 and 61.9 ppm which were characteristic of acetoxy methylene moieties. The position of the remaining 14 signals between 50 and 16 ppm (with the exception of the C 5 signal at 35.2 ppm) were almost identical to the corresponding carbon resonances in **(6)** and the other novel monocyclic metabolites isolated from *C. trifaria* (Chapter 3).

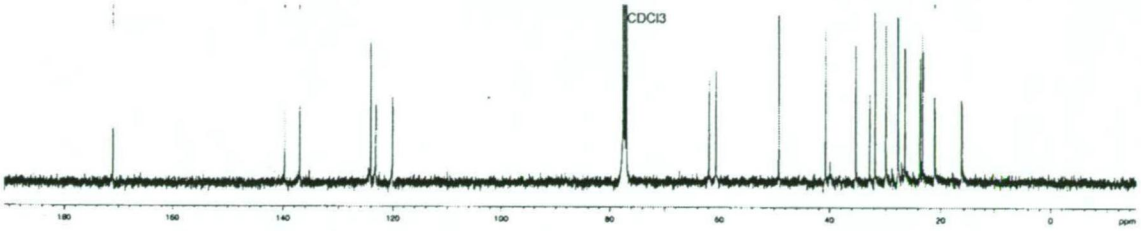


Figure 4.17 ^{13}C n.m.r. spectrum of (11) (400MHz, $CDCl_3$)

The multiplicity of the protonated carbons of **(11)** was determined by a DEPT experiment (Appendix 2, Figure 2.7); indicating that **(11)** possessed four methine, eight methylene and six methyl groups. The six remaining ^{13}C resonances at 171.0, 170.9, 139.6, 136.9, 136.9 and 32.7 ppm were therefore attributed to quaternary carbon atoms (Table 4.2). A GHMQC n.m.r. experiment (Figure 4.18) provided information on the ^{13}C-^{1}H connections in **(11)** (Table 4.2) whilst a COSY n.m.r. experiment (Figure 4.19) provided information on the ^{1}H-^{1}H couplings in **(11)**. In conjunction with a HMBC n.m.r. experiment (Figure 4.20) the COSY n.m.r. experiment was used to determine the structure of **(11)**.

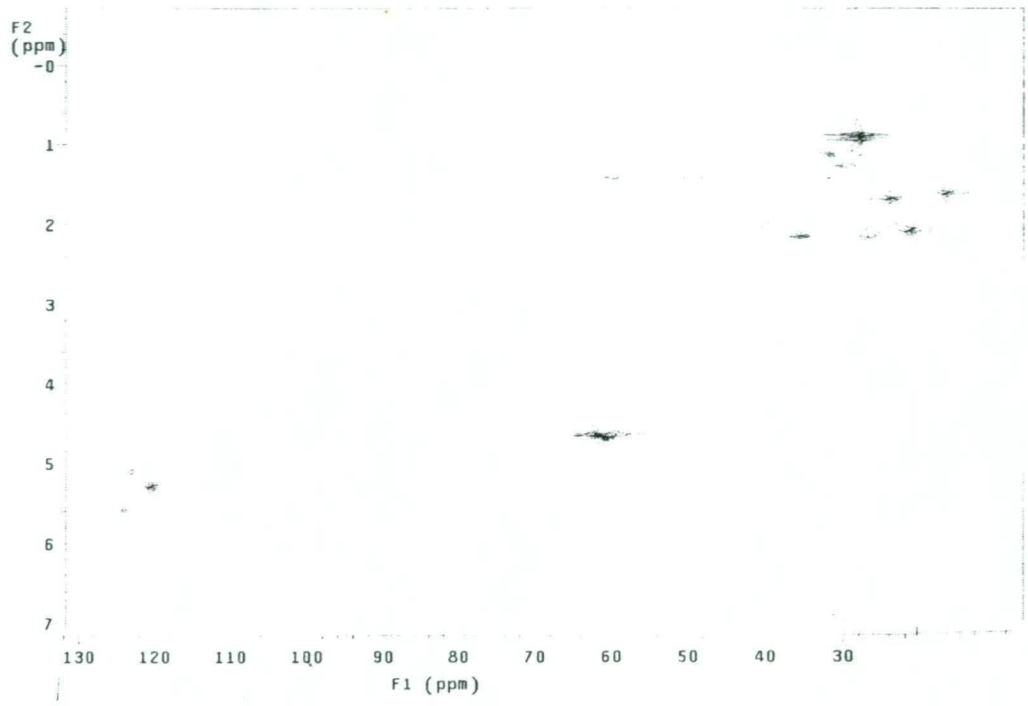
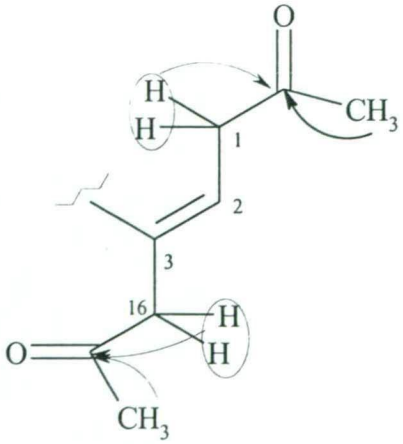


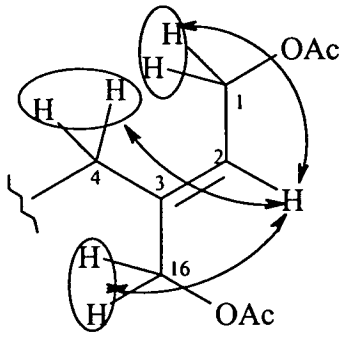
Figure 4.18 GHMQC n.m.r. spectrum of (11) (100MHz, $CDCl_3$)

The HMBC n.m.r. experiment showed connections from the methyl group protons signals at 2.05 and 2.07 ppm to the carbonyl carbon signals at 170.9 ppm and 171.0 ppm and also from the H 1 and H 16 methylene proton signals at 4.66 ppm and 4.63 ppm to these carbonyl carbon signals at 170.9 ppm and 171.0 ppm.

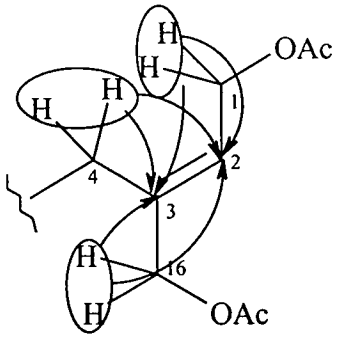


The COSY n.m.r. experiment (Figure 4.20) showed coupling between the H 1 methylene protons at 4.66 ppm and the H 2 methine proton at 5.56 ppm. This H 2 proton at 5.56

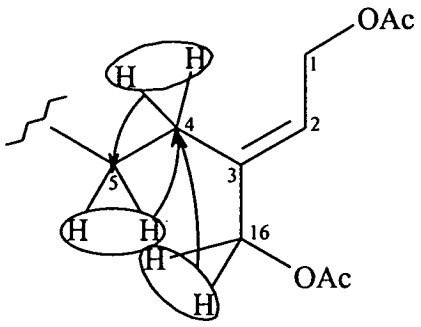
ppm also showed long range coupling (^4J) to the H 16 methylene protons at 4.63 ppm and the H 4 methylene protons at 2.13 ppm.



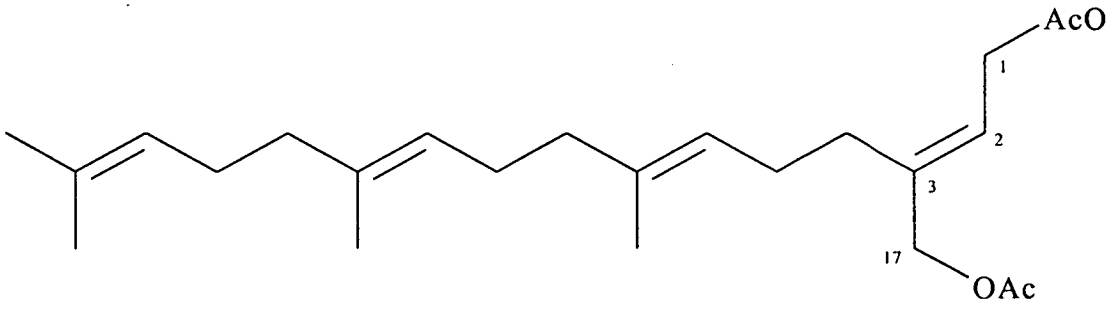
Furthermore the H 1, H 16 and H 4 protons at 4.66 ppm, 4.63 ppm and 2.13 ppm respectively, showed HMBC correlations to the quaternary C 3 signal at 136.9 ppm and the C 2 signal at 124.1 ppm.



The signals for the H 4 and H 5 methylene protons were both in the region 2.10-2.13 ppm; this overlap making it difficult to see a COSY connection. The HMBC experiment however showed a connection between the H 5 methylene protons at 2.10 ppm and the C 4 signal at 35.2 ppm and also between the H 4 methylene protons at 2.13 ppm.and the C 5 signal at 26.4 ppm The H 16 methylene protons at 4.63 ppm also showed a HMBC connection to the C 4 signal at 35.2 ppm.



A compound **(13)** with a similar functionality was isolated in 1990 by Wright and Coll from the tropical green alga *Chlorodesmis fastigiata* off the North Queensland coast.[8] It can be seen from Table 4.3 that the ^{1}H n.m.r. data for the C 1, C 2 and C 16/17 protons of **(11)** and **(13)** are identical.



(13)

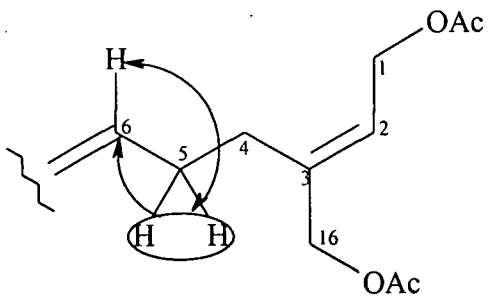
The steroechemistry of the C 2-C 3 double bond in **(13)** was determined to be *E* on the basis of n.O.e experiments.[8] Hence the stereochemistry of the C2-C 3 double bond in **(11)** was also determined to be *E* since its ^{1}H n.m.r. data are identical to that of **(13)**. The novel monocyclic metabolite identified from *C. trifaria* (Chapter 3, Section 3.6) possesses the *Z* stereochemistry about the C 2-C 3 double bond.

Carbon no.	(13)	(11)
1	4.66	4.66
2	5.56	5.56
16/17	4.63	4.63

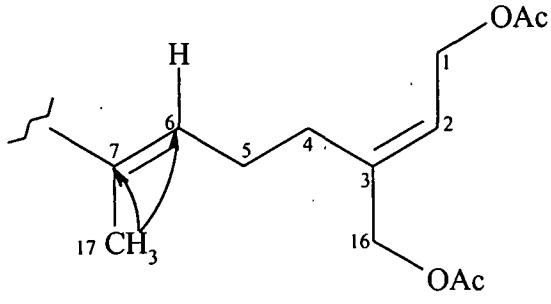
Table 4.3 Partial ^{1}H n.m.r. data for (11) and (13) (ppm, $CDCl_3$)

The COSY and HMBC connections for the remainder of **(11)** are as follows:

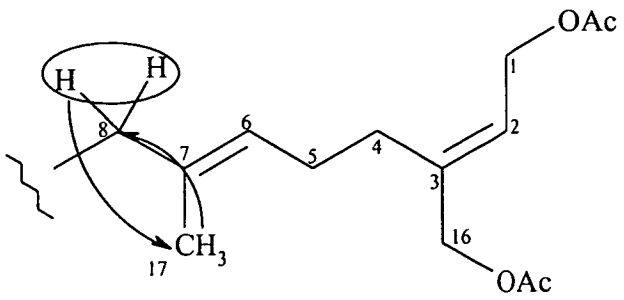
The H 5 methylene protons at 2.10 ppm showed a COSY connection to the H 6 methine proton at 5.08 ppm and a HMBC connection to the C 6 signal at 123.1 ppm.



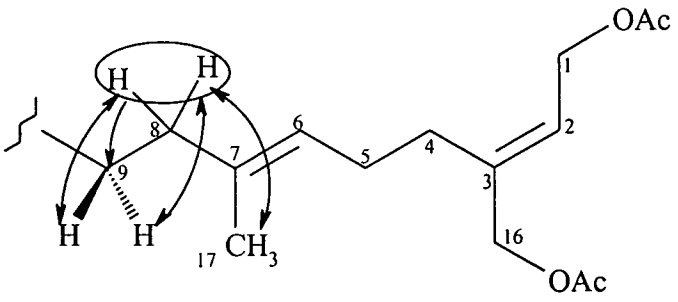
Furthermore the H 17 methyl protons at 1.60 ppm showed HMBC correlations to the C 6 signal at 123.1 ppm and the C 7 signal at 136.9 ppm.



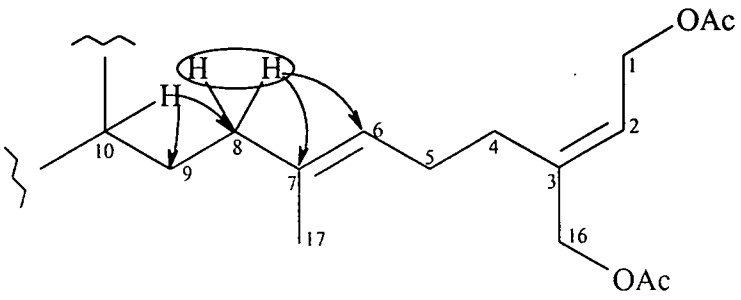
The H 17 methyl protons at 1.60 ppm in turn showed a HMBC connection to the methylene C 8 signal at 40.7 ppm whose protons at 1.96 ppm showed a HMBC connection to the C 17 signal at 16.26 ppm.



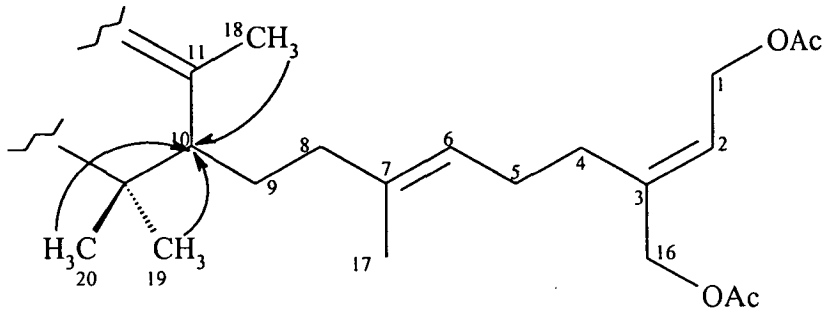
These H 8 protons at 1.96 ppm in turn showed a HMBC connection to the C 9 signal at 30.0 ppm and COSY connections to the H 9 protons at 1.50 and 1.34 ppm and the H 17 methyl protons at 1.60 ppm.



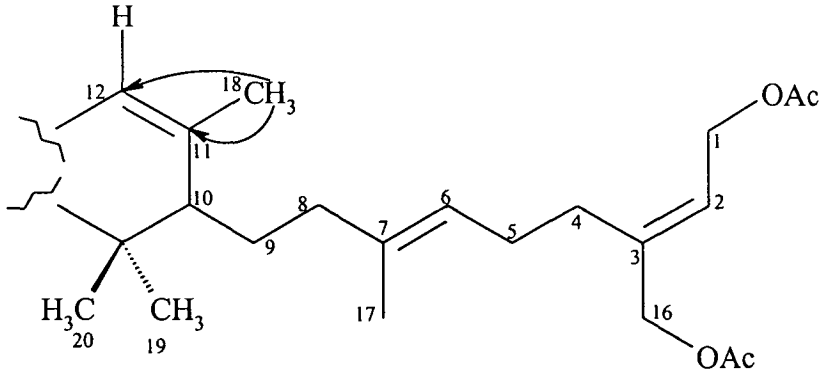
The H 8 methylene protons at 1.96 ppm also showed HMBC correlations to the C 7 signal at 136.9 ppm and the C 6 signal at 123.16 ppm whilst the H 10 methine proton at 1.40 ppm showed HMBC correlations to the C 9 signal at 30.0 ppm and the C 8 signal at 40.7 ppm.



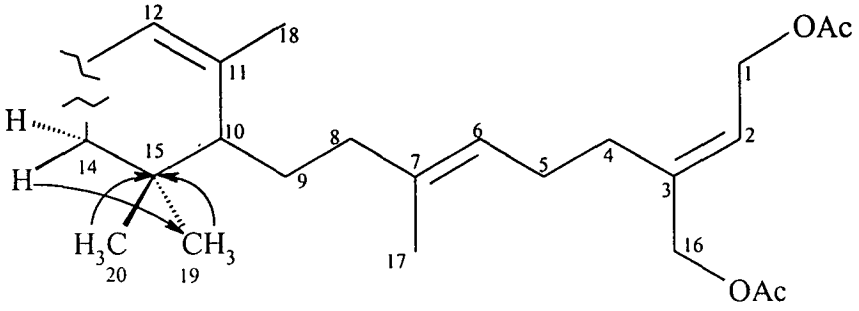
Furthermore the H 19 and H 20 dimethyl proton signals at 0.86 and 0.92 ppm and the H 18 methyl protons at 1.60 ppm showed HMBC connections to the C 10 signal at 49.2 ppm



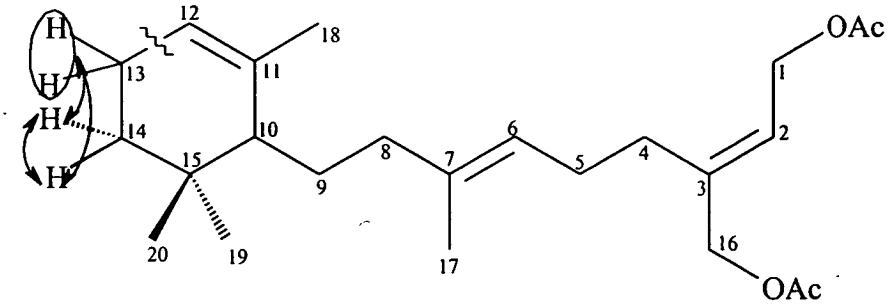
The H 18 methyl protons at 1.66 ppm also showed HMBC connections to the C 12 signal at 120.0 ppm and the C 11 quaternary carbon signal at 136.9 ppm.



The H 19 and H 20 methyl group protons at 0.86 ppm and 0.92 ppm showed HMBC correlations to the C 15 signal at 32.7 ppm whilst the H 14 proton at 1.42 ppm showed a HMBC correlation to the C19 and C 20 signals at 27.7 and 27.6 ppm.

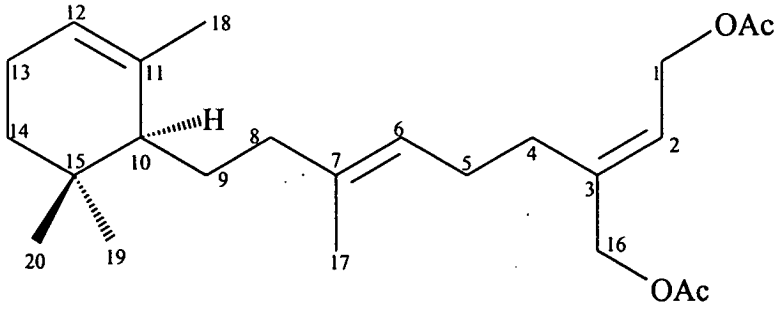


The H 13 protons at 1.96 ppm showed a COSY connection to the H 14 protons at 1.42 ppm and 1.10 ppm. The H 14 proton at 1.10 ppm also showed a COSY connection to its non-equivalent counterpart at 1.42 ppm



Whilst the H 12 methine proton at 5.28 did not show a COSY correlation to the H 13 protons at 1.96 ppm, a bond between C 12 and C 13 is consistent with all the spectral eveidence presented so far.

The absolute stereochemistry of the chiral centre at C 10 was assigned as *S* based on the negative sign for the optical rotation of **(11)**. The reasons for this assignment have been discussed in Chapter 3. Thus the evidence indicates that the structure of **(11)** is as shown below:



(11)

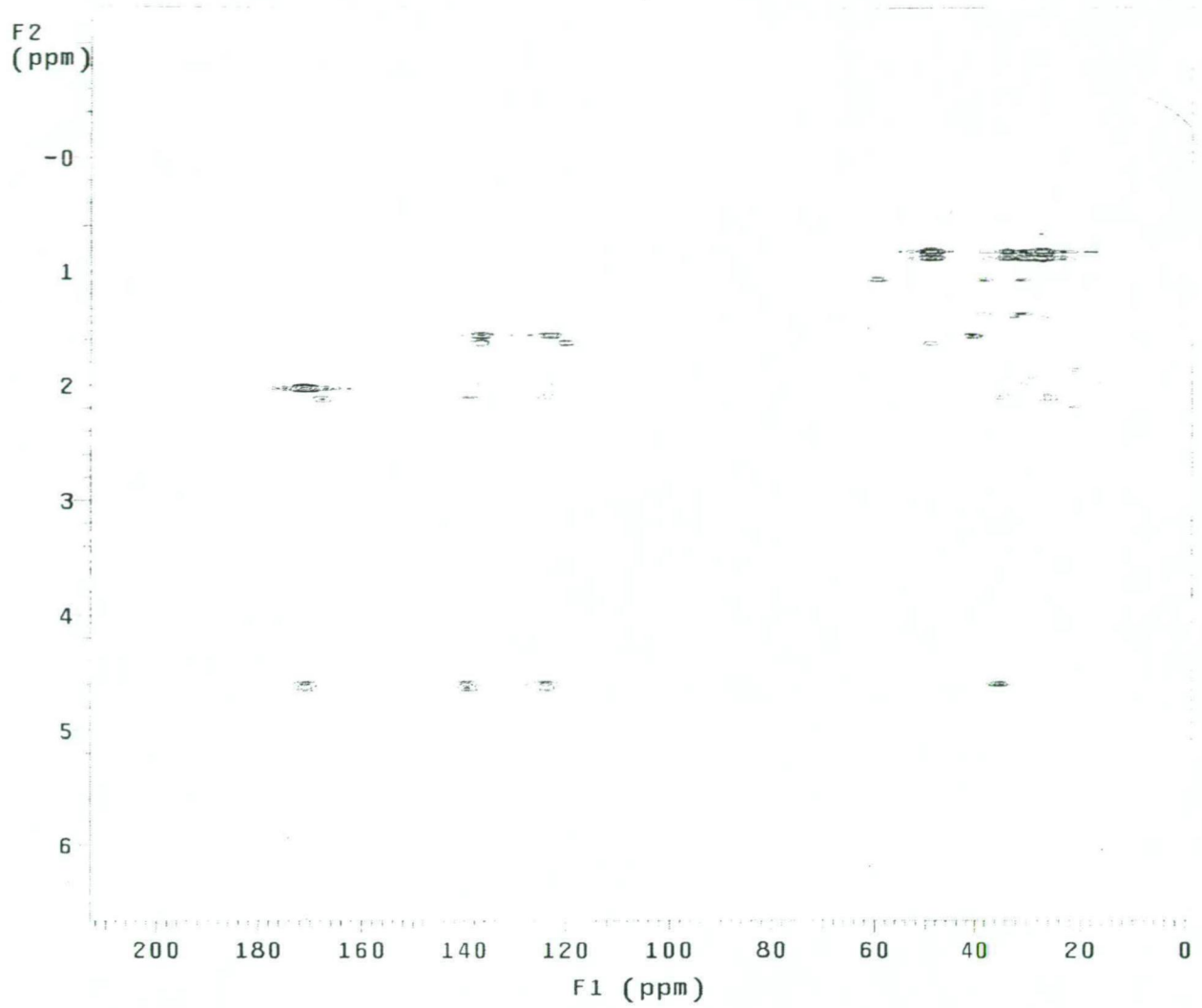


Figure 4.19 HMBC n.m.r. spectrum of (11) (100MHz, $CDCl_3$)

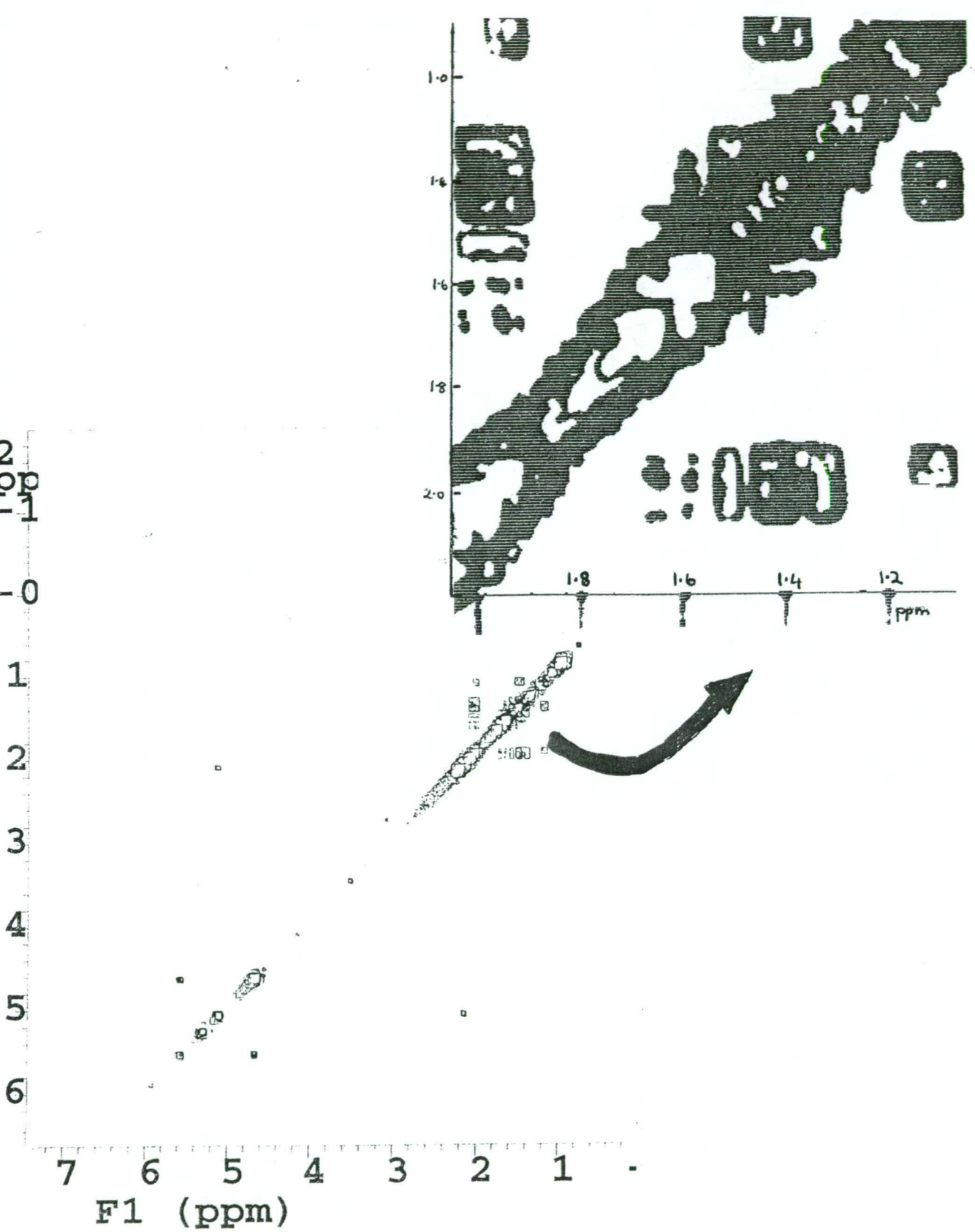


Figure 4.20 COSY n.m.r. spectrum of (11) (400MHz, $CDCl_3$)

The second fraction obtained by h.p.l.c. possessed the g.c. profile shown in the lower half of Figure 4.21; (the upper g.c. profile is that of **(11)**).

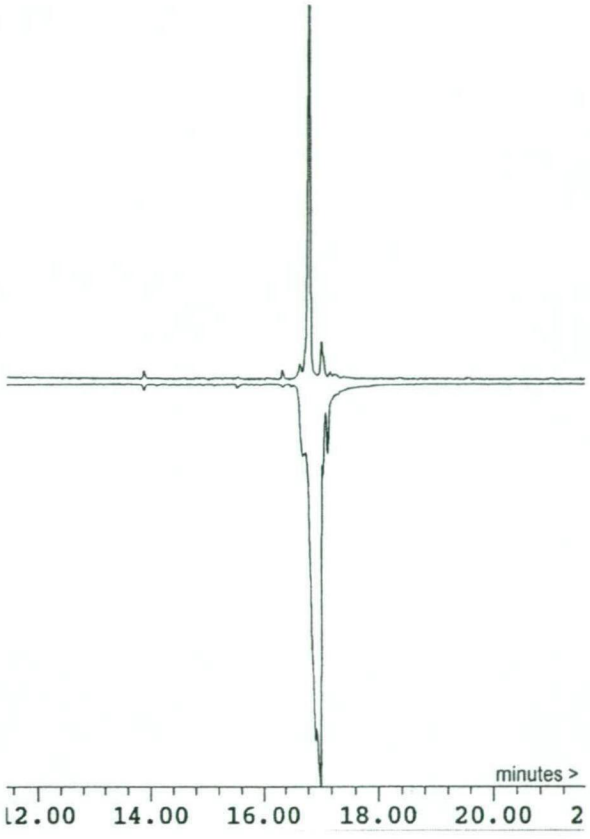
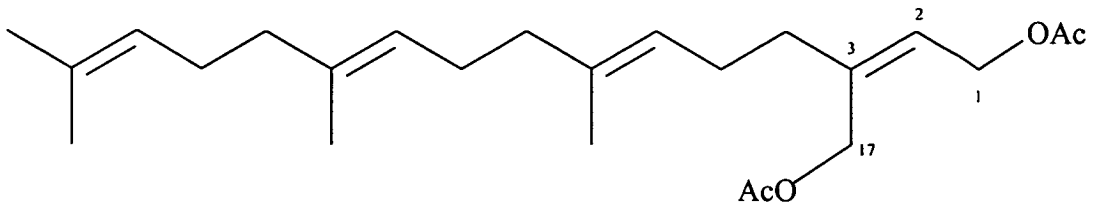


Figure 4.21 G.c. profile of (11) (upper) and (12) (lower)

The three minor peaks present in the g.c. of **(12)** all possessed 390 as the highest molecular ion with peaks at *m/z* 330 and 270 consistent with the presence of two acetoxy groups. One of these minor peaks showed ions at 149 and 191 a.m.u. consistent with the acyclic structure shown in didehyrotrifarin **(5)** and trifarin **(4)**; however this spectrum did not match the mass spectrum of either of these two metabolites. It also did not match the mass spectrum of **(13)** given in the literature.[8] The ^{1}H n.m.r. spectrum of this fraction showed small signals typical of the *Z* isomer (Figure 4.18) and thus the presence of a

metabolite with the structure **(14)** would be consistent with the m.s. and ^{1}H n.m.r. spectral data of this minor metabolite.



(14)

The m.s. data of the other two minor metabolites were different to those of either the cyclic or acyclic metabolites described previously. The mass spectrum of the major peak present in **(12)** was identical to that of **(11)** whilst the second most abundant peak showed two small differences (in the *m/z* clusters at 69 and 107 units). The mass spectra for these two metabolites showed the fragmentation pattern typical of a 1,3,3-trimethyl substituted cyclohexene and also possessed 390 as the highest molecular ion with *m/z* peaks at 330 and 270 consistent with the presence of two acetoxy groups.

The ^{1}H n.m.r. spectrum of **(12)** was almost identical to that of **(11)** showing a high degree of purity with small signals due to a *Z* isomer (C 2-C 3) at 4.54 ppm and 5.60 ppm (Figure 4.22).

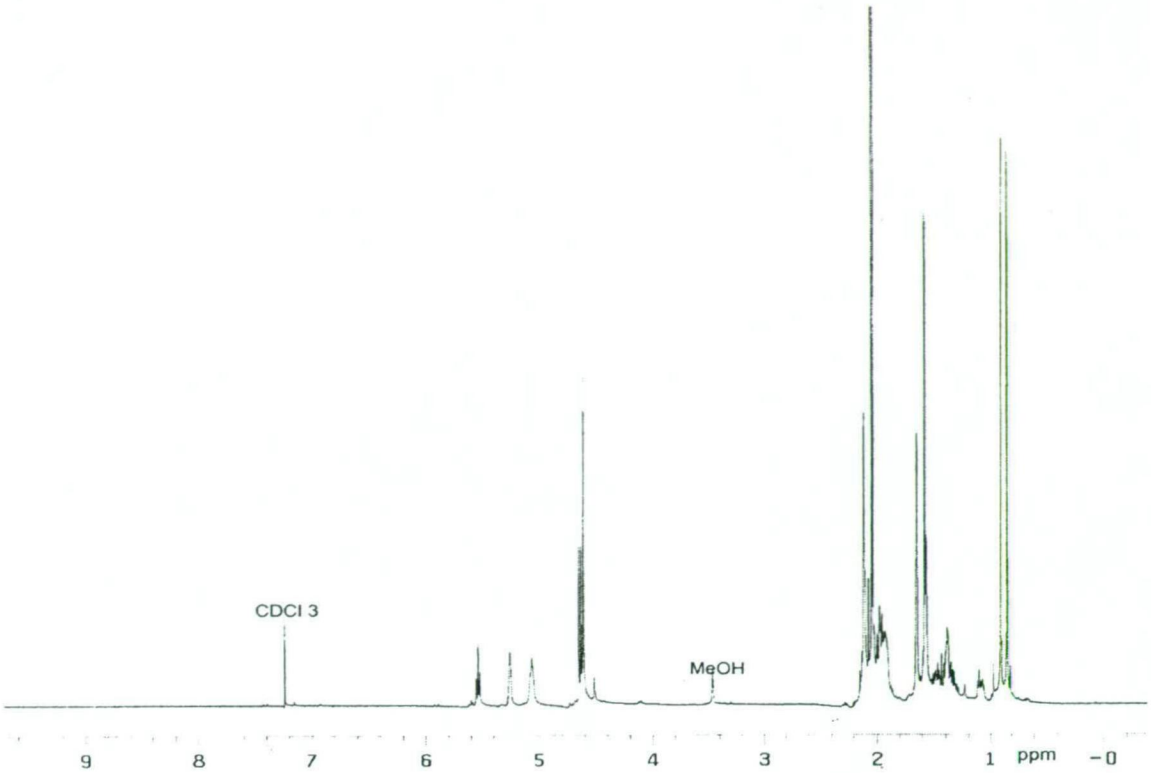


Figure 4.22 ^{1}H n.m.r. spectrum of (12) (400MHz, $CDCl_3$)

The ^{13}C n.m.r. spectrum of **(12)** (Figure 4.23) indicated that this fraction consisted of one major metabolite with spectral data identical to that of **(11)** (Table 4.2).

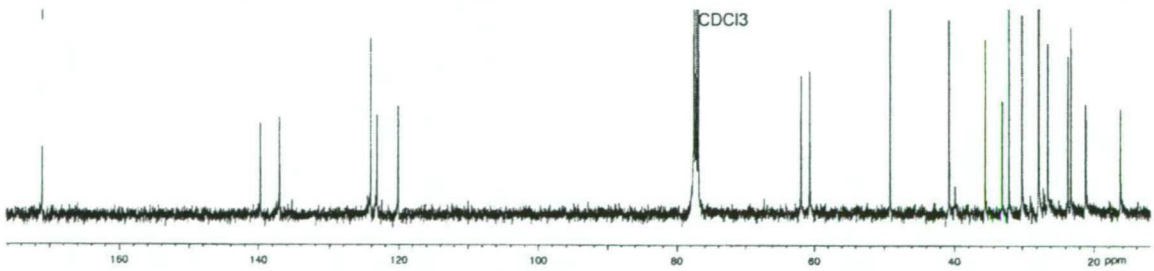


Figure 4.23 ^{13}C n.m.r. spectrum of (12) (100MHz, $CDCl_3$)

The DEPT and COSY n.m.r. spectra of **(12)** are contained in Appendix 2 (Figures 2.9-2.10). A detailed examination of these spectra and a HMBC spectrum of **(12)** resulted in the elucidation of an identical structure to **(11)** with COSY n.m.r. correlations being present this time for protons attached to C 12 and C 13.

The u.v. spectra of the **(11)** and **(12)** showed some differences however no conclusions could be drawn from these differences except that the u.v. spectrum of **(12)**

was consistent with the elucidated structure whilst that of **(11)** contained an extra unusual peak (which remained on a repeat spectrum with a different part of the sample). The optical rotations of the fractions **(11)** and **(12)** were negative and of approximately the same magnitude.

The conclusion that can be drawn from the foregoing discussion is that the major metabolites in fractions **(11)** and **(12)** appear to possess the same structure. It is possible that the presence of minor metabolites in **(12)** with different u.v. characteristics resulted in some of metabolite **(11)** being collected into fraction **(12)**. However this does not explain the identical n.m.r. data for the two fractions as the major metabolite in fraction **(12)** was not metabolite **(11)** but another metabolite with almost identical m.s. but a different retention time. The employment of more sophisticated n.m.r. techniques than used in this investigation may be able to determine the difference in structure between the two metabolites. Furthermore if the purification procedure was repeated, a refinement of the h.p.l.c. techniques used (based on experience gained on subsequent seaweeds) should further improve the separation of the metabolites present in this p.t.l.c. fraction.

	(11)		(12)	
Carbon	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	60.6 t	4.66 d *J* 6.9 Hz	60.6 t	4.66 d *J* 6.9 Hz
2	124.1 d	5.56 t *J* 6.9 Hz	124.1 d	5.56 t *J* 6.9 Hz
3	136.9 s	------	136.9 s	------
4	35.2 t	2.13 m	35.2 t	2.15 m
5	26.5 t	2.10 m	26.5 t	2.10 m
6	123.1 d	5.08 br s	123.1 d	5.08 br s
7	136.9 s	------	136.9 s	------
8	40.7 t	1.96 br s	40.7 t	1.96 br s
9	30.0 t	1.50/1.34 m	30.0 t	1.50/1.34 m
10	49.2 d	1.40 m	49.2 d	1.40 m
11	136.9 s	------	136.9 s	------
12	120.0 d	5.28 br s	120.0 d	5.28 br s
13	23.3 t	1.96 br s	23.3 t	1.96 br s
14	31.8 t	1.42/1.10 m	31.8 t	1.42/1.10 m
15	32.7 s	--------	32.7 s	--------
16	61.9 t	4.63 s	61.9 t	4.63 s
17	16.3 q	1.60 s	16.3 q	1.58 s
18	23.7 q	1.66 s	23.7 q	1.60 s
19	27.6/7 q	0.86 s	27.6/7 q	0.86 s
20	27.6/7 q	0.92 s	27.6/7 q	0.92 s
$O\underline{C}OCH_3$	171.0, 170.9 s	-----	171.0, 170.9 s	-----
$OCO\underline{C}H_3$	21.14. 21.18 q	2.05, 2.07 s	21.14. 21.18 q	2.05, 2.07 s

Table 4.2 ^{1}H and ^{13}C n.m.r data for (11) and (12) ($CDCl_3$)

4.8 Structure elucidation of metabolites (15) and (16)

The next most polar fraction obtained by p.t.l.c. was subjected to normal phase h.p.l.c (SiO_2, 8 % EtOAc in pet. ether) to yield a further three fractions. The l.c.-m.s. of the major and least polar fraction indicated that two similar compounds of molecular weight of 404 a.m.u. were present corresponding to a molecular formula of $C_{24}H_{36}O_5$. Furthermore the mass spectral data of this fraction showed a peak at *m/z* 344 a.m.u. (loss of 60 =AcOH) suggesting that an acetoxy group was present which was further supported by an absorption band at 218 nm (ε=6050, EtOH) in the u.v. spectrum.

The ^1H n.m.r. spectrum of this fraction (Figure 4.24) included a signal at 9.31 ppm typical of an aldehyde group and a doublet at 7.44 ppm coupled to a doublet at 5.49 ppm. Other signals of note were two sharp singlets at 2.15 ppm and 2.17 ppm typical of acetoxy methyl groups and signals in the 0.85 ppm to 2.0 ppm region typical of both the acyclic and monocyclic metabolites ((**5**) and (**6**), (**7**) and (**8**)) found in previous fractions in *C. brownii.*

Intensive efforts over six months and four collections to separate the two metabolites in this fraction by normal and reverse phase h.p.l.c. were ultimately unsuccessful. A similar method to that used in separating the acyclic and monocyclic metabolites (**7**) and (**8**); (5 % H_2O in acetonitrile, reverse phase C 18 analytical column, dilute injections) initially appeared to be successful giving a pure compound of molecular weight of 404 a.m.u. by l.c.-m.s. however when enough material had been accumulated for ^1H n.m.r studies it was found that the metabolite present was a 1,4-acetoxybutadiene; suggesting that the original compound had decomposed or

reacted with the water in the solvent mixture. (Throughout the investigations undertaken for this thesis several metabolites were found to be unstable on h.p.l.c. with solvent mixtures containing water.)

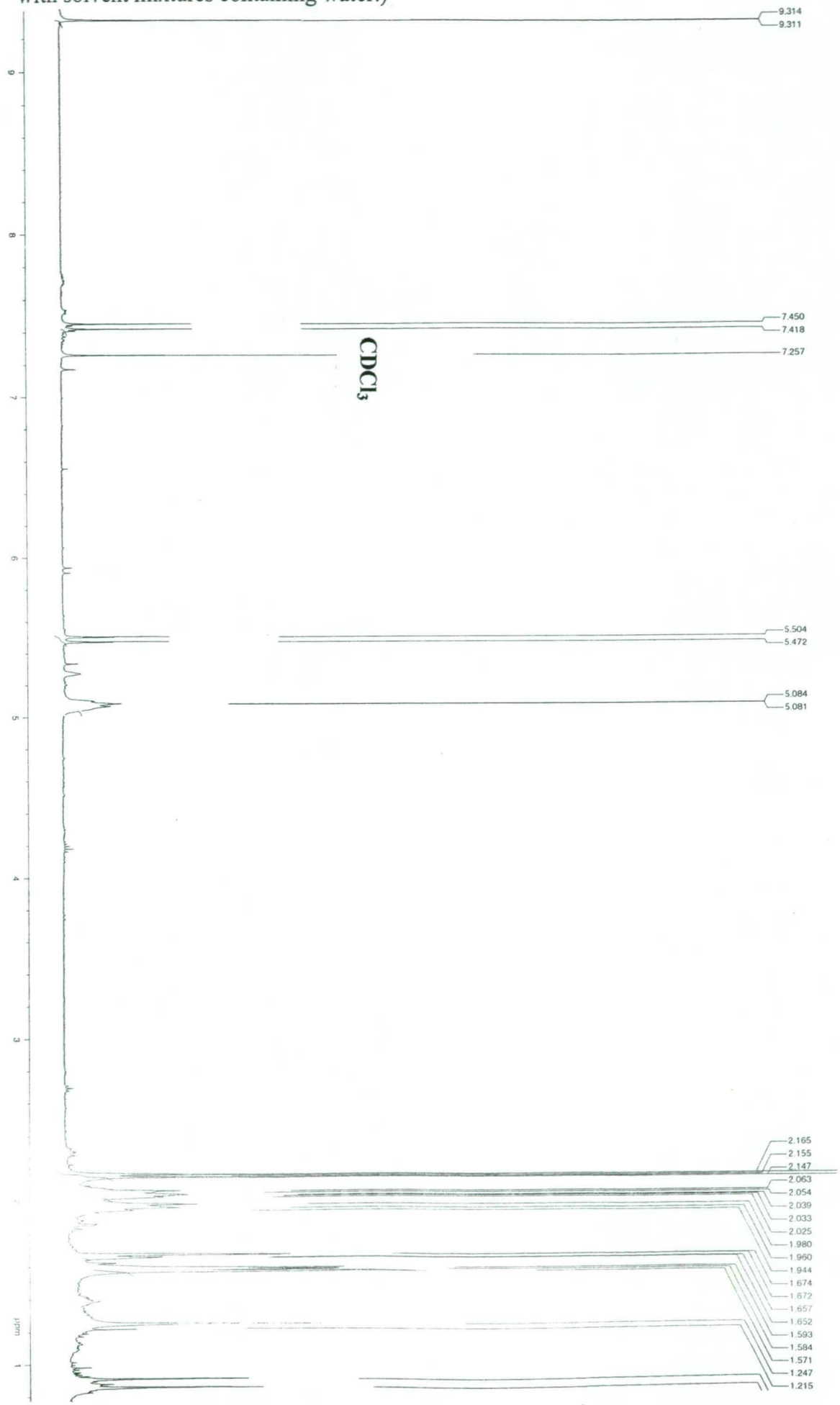


Figure 4.24 ^{1}H n.m.r. spectrum of (15) and (16) (400MHz, $CDCl_3$)

The ^{13}C n.m.r. spectrum of **(15)** and **(16)** (Figure 4.25) contained a major and a minor set of twenty four resonances; with eight of these resonances being common; 195.1, 170.3 167.8, 138.8, 110.3, 26.8, 21.0 and 84.8 ppm (Table 4.4). The remaining signals were identical to either the monocyclic or acyclic C 5 – C 20 moieties identified in previous fractions of *C. brownii* (unbranched).

The multiplicity of the protonated carbons of **(15)** and **(16)** were determined by a DEPT experiment; indicating that each possessed six methine, eight methylene and six methyl groups. The remaining ^{13}C resonances at 170.3, 167.8, 139.7, 136.8, 136.9, 135.3, 131.5, 84.8 and 32.7 ppm were therefore attributed to quaternary carbon atoms (Table 4.4).

A GHMQC n.m.r. experiment provided information on the ^{13}C-^{1}H connections for **(15)** and **(16)** whilst a COSY n.m.r. experiment provided information on the ^{1}H-^{1}H couplings. In conjunction with a HMBC n.m.r. experiment the COSY experiment was used to determine the structure of **(15)** and **(16)**.

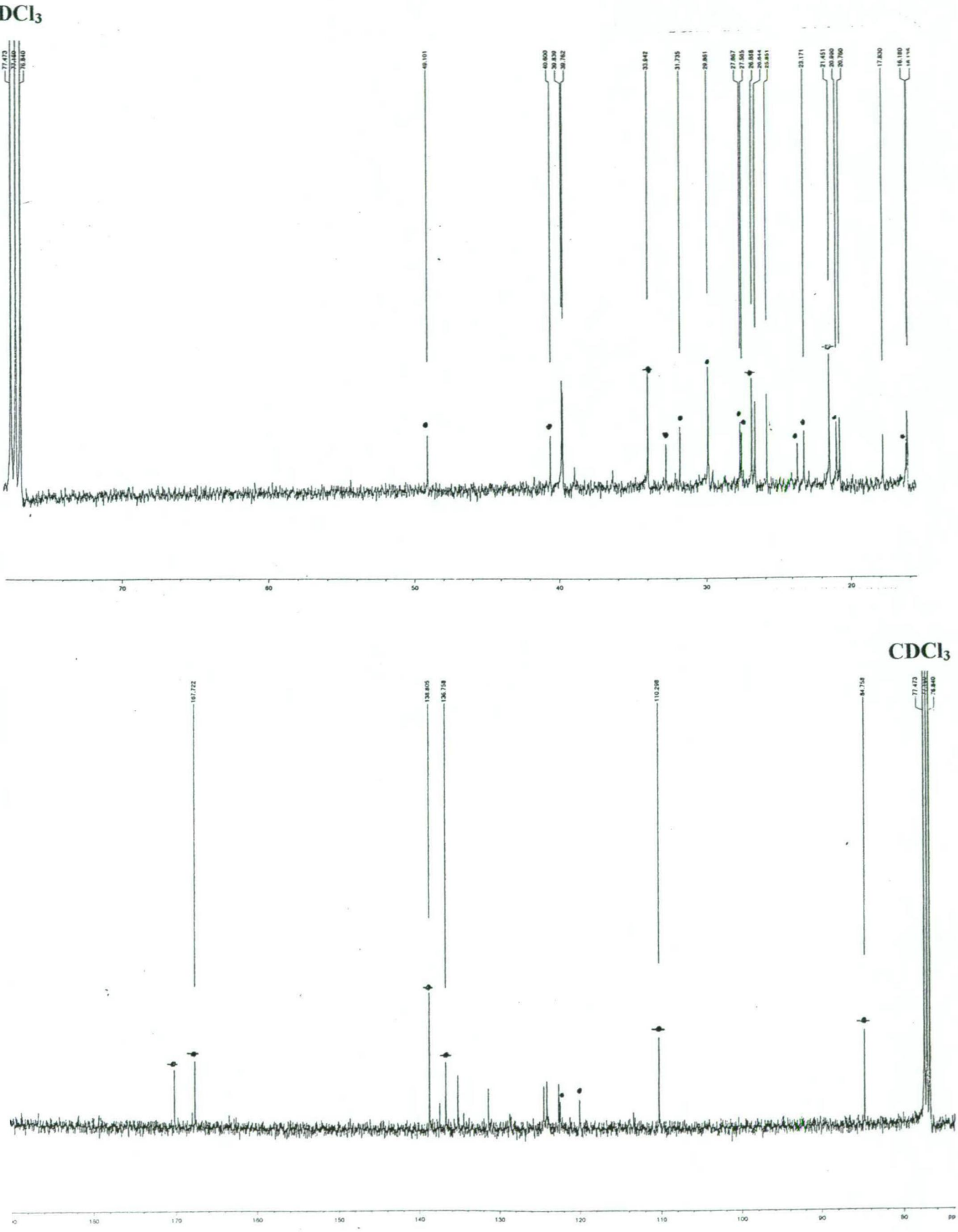


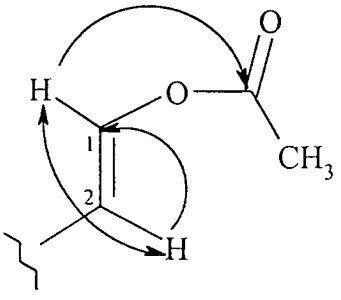
Figure 4.25 ^{13}C n.m.r. spectrum of (15) and (16) (100MHz, $CDCl_3$)

● = minor (monocyclic), ●- = common peaks

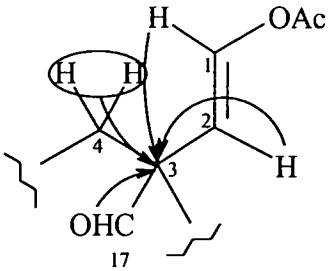
	(15)				(16)			
C	^{13}C	^{1}H	HMBC	COSY	^{13}C	^{1}H	HMBC	COSY
1	138.8	7.44	H 2	H 2	138.8	7.44	H 2	H 2
2	110.3	5.49		H 1	110.3	5.49		H 1
3	84.8	------	H1,2,4,17	----	84.8	------	H1,2,4,17	----
4	34.0	1.9			34.0	1.9		
5	26.9	1.94-2.06	H 6		26.9	1.94-2.06	H 6	
6	124.5	5.08		H 18	122.5	5.08		H 18
7	136.8	----	H 18	-----	136.8	----	H 18	-----
8	39.9	1.94-2.06	H 9, H 18	H 18	40.6	1.9	H 9	H 9
9	26.7	1.94-2.06	H 10	H 10	29.9	1.3		H 8, H 10
10	124.1	5.08		H 19	49.1	1.4	H 19, H 20	H 19, H 20
11	135.3	------	H 19		136.9	------		
12	39.8	1.94-2.06	H13, H 19		120.1	5.28		H13, H 18
13	21.5	1.94-2.06	H 14		23.2	1.96		H 12, H 14
14	122.6	5.08			31.8	1.42/1.10		H 13
15	131.5	------	H16, H 20		32.7	--------		
16	25.9	1.6		H 6, H 8	195.1	9.31		
17	195.1	9.31			16.3	1.58		H 6
18	16.2	1.65		H 6, H 8	23.6	1.59		H12, H 13
19	17.8	1.66		H8, H 10	27.6/7	0.85		H 10
20	16.1	1.67		H 14	27.6/7	0.90		H 10
	167.8		H 1		167.8		H 1	
	170.3				170.3			
	21.1/.2	2.16/2.14			21.1/.2	2.17/2.15		

Table 4.4 ^{1} H, ^{13}C, HMBC and COSY n.m.r data for (15) and (16) (ppm, $CDCl_3$)

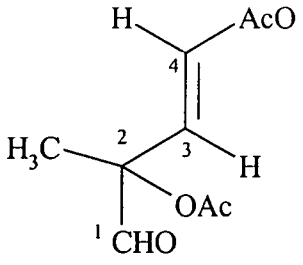
The COSY n.m.r. experiment (Figure 4.26) showed a correlation between the H 1 proton at 7.44 ppm and the H 2 proton at 5.49 ppm whilst the HMBC n.m.r. experiment (Figure 4.27) showed a correlation between the H 1 proton signal at 7.44 ppm and the carbonyl carbon signal at 167.8 ppm and also the H 2 proton signal at 5.49 ppm and the C 1 signal at 138.8 ppm.



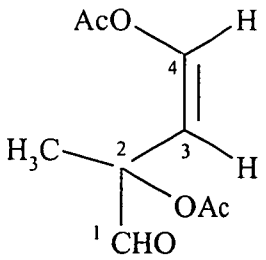
The H 1 proton signal at 7.44 ppm, the H 2 proton signal at 5.44 ppm, the H 17 aldehyde proton signal at 9.32 ppm and the H 4 proton signal at 1.9 ppm showed HMBC connections to the quaternary carbon signal at 84.8 ppm (C 3)



Compounds with an acetoxy group attached to C 3 have been synthesised by Fischer, Krapf and Paust[9] in 1988 as valuable intermediates in the synthesis of terpenes such as retinal, beta-carotene and apocarotinoids. A mixture of compound **(17)** (*E*)-2-methyl-2,4-diacetoxy-but-3-enal and its *Z* isomer **(18)** was isolated and identified by ^{1}H n.m.r.[9]



(17)



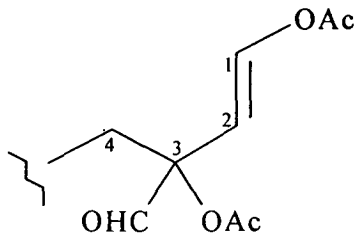
(18)

The ^{1}H n.m.r data for compounds **(17)**, **(18)** and the metabolites **(15)** and **(16)** obtained from *C. brownii* are shown below in Table 4.5.

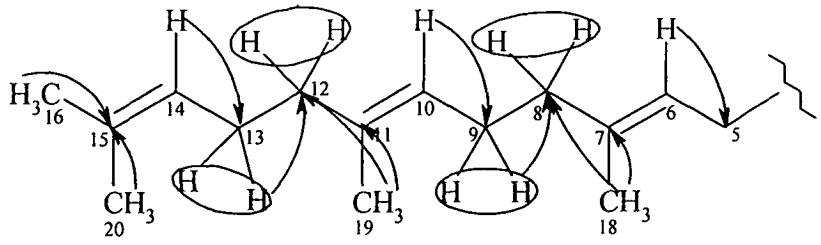
Carbon no.	(17)	(18)	(15)	(16)
1	9.28	9.37	9.31	9.31
3	5.48	5.15	5.49	5.49
4	7.41	7.10	7.44	7.44

Table 4.5 Partial ^{1}H n.m.r data for compounds (15)-(18)[9](ppm),($CDCl_3$)

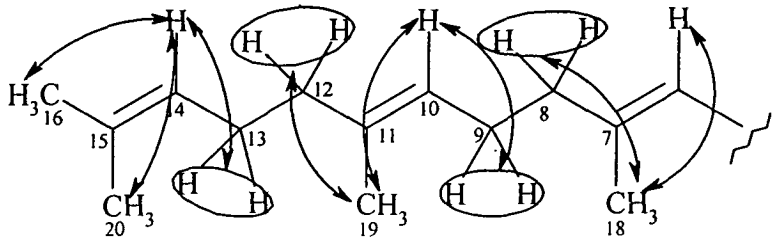
It can be seen from Table 4.5 that the data for the *E* isomer **(17)** is almost identical to the corresponding moiety in **(15)** and **(16)**. Thus the evidence so far supports the inclusion of the C 1-C 4 moiety (below) in the structures of **(15)** and **(16)**. This was further supported by comparison with simulated ACDLabs data for this C 1 to C 4 moiety.



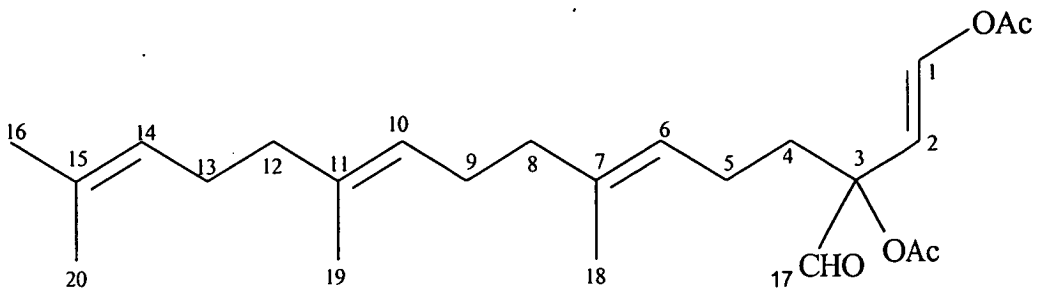
The HMBC correlations for C 5 to C 20 (Table 4.4) for the major metabolite **(15)** are shown below:



The COSY n.m.r. correlations for the substructure C 6 to C 20 of **(15)** (shown below) are similar to the COSY n.m.r. correlations for the other metabolites **(5)**, **(8)** and **(13)** possessing the acyclic substructure:

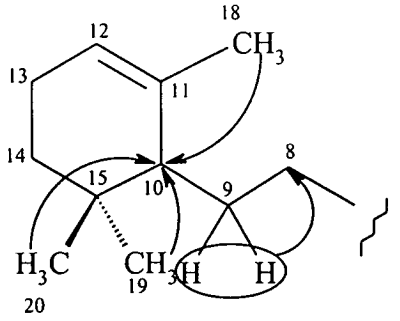


Hence the evidence presented supports the linking of the two substructures present in the major metabolite **(15)** to give the final structure of **(15)** as:

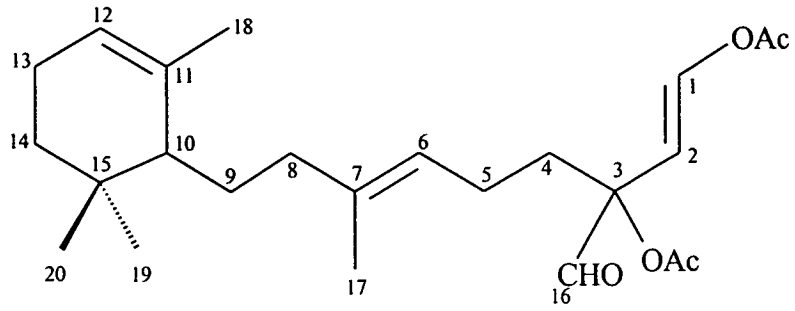


(15)

The HMBC and COSY n.m.r. data (Table 4.4) also included signals (shown below) similar to those found in the monocyclic metabolites previously isolated from *C. trifaria* and previous fractions of *C. brownii* (unbranched).



Thus the spectral evidence presented here is consistent with the following structure for the minor metabolite **(16)** present in this fraction:



(16)

.The optical rotation of the fraction containing **(15)** and **(16)** in the ratio 80:20 was found to be negative. Due to the small quantities isolated no attempt was made to determine the absolute configuration of the C 3 moiety

The presence of the both monocyclic and acyclic metabolites appears to be a feature of *C. brownii* (unbranched). While these metabolites were able to be separated in the fraction containing **(7)** and **(8)**, the instability of the metabolites containing the C 1 –C 4 moiety prevented separation in this case. During investigations for this thesis an acyclic metabolite containing an identical C 1 to C 4 substructure was subsequently found in *C. flexilis* in larger amounts and this moiety will be discussed further in Chapter 5.

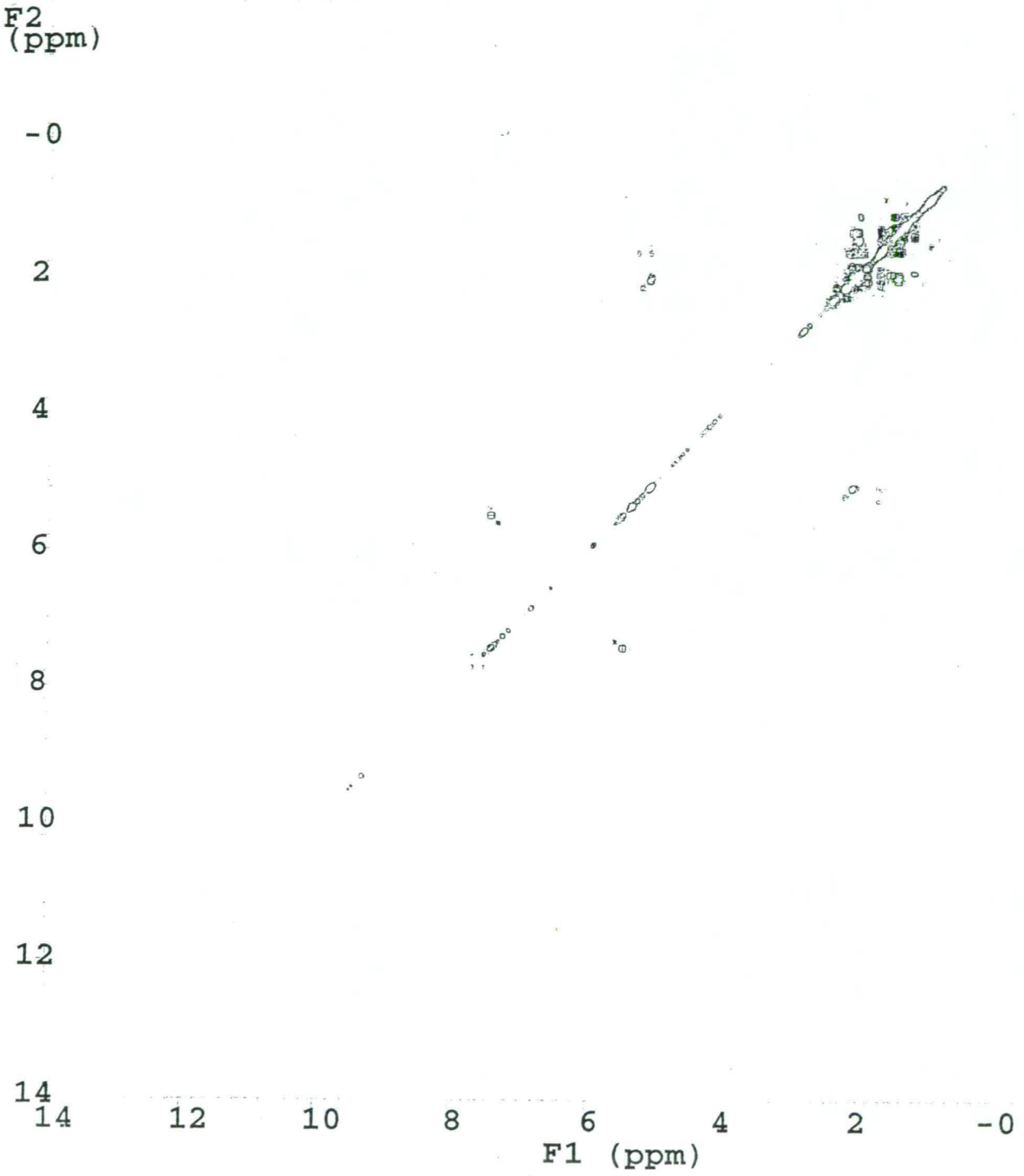


Figure 4.26 COSY n.m.r. spectrum of (15) and (16) (400MHz, $CDCl_3$)

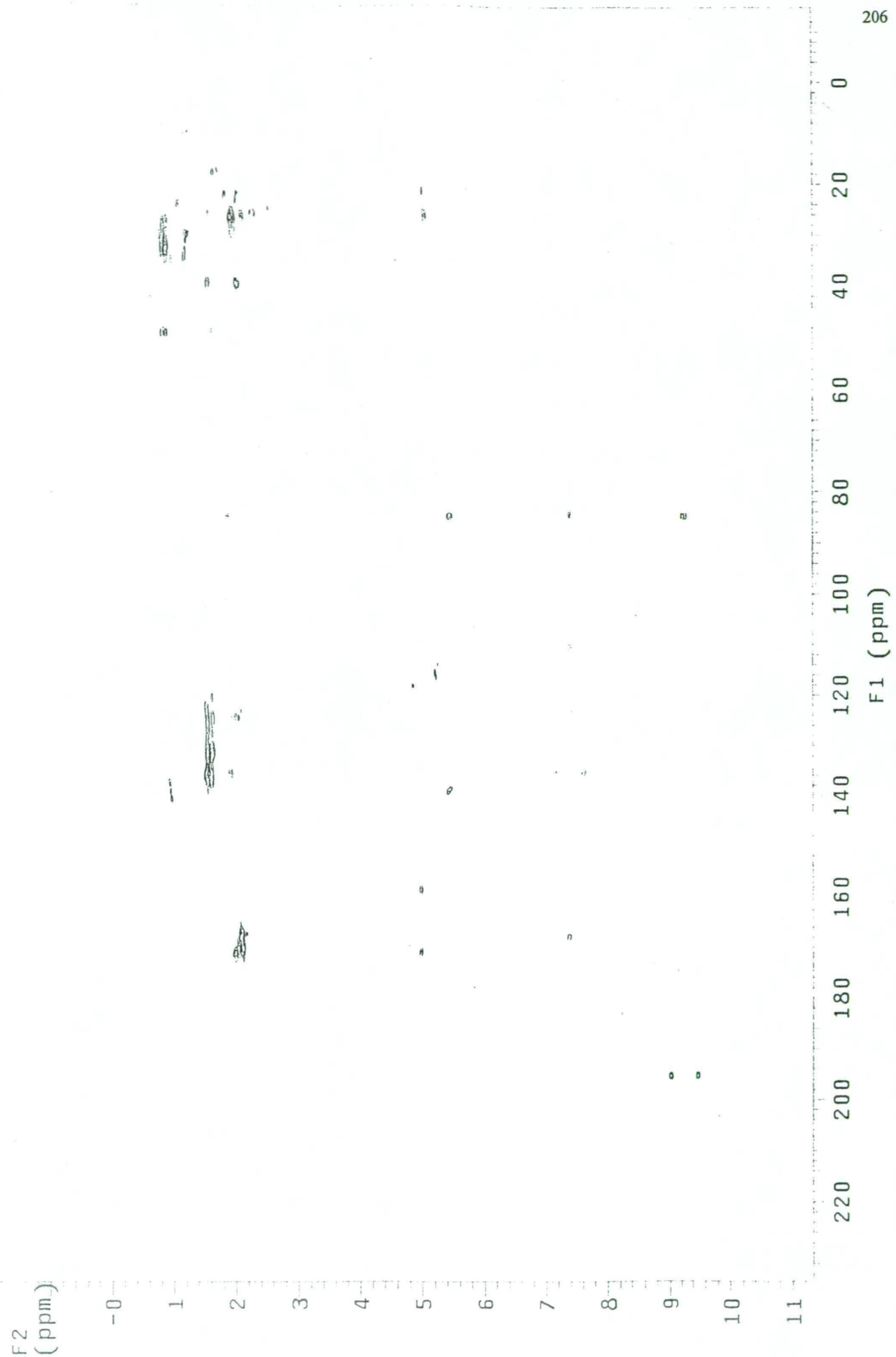


Figure 4.27 HMBC n.m.r. spectrum of (15) and (16) ($CDCl_3$)

4.9 Structure elucidation of metabolites (19) and (20)

The remaining two minor fractions obtained after h.p.l.c. consisted of a fraction with similar ^{1}H n.m.r. data to those of **(15)** and **(16)** which decomposed on further h.p.l.c. and a third fraction whose ^{1}H n.m.r. spectrum is shown in Figure 4.28.

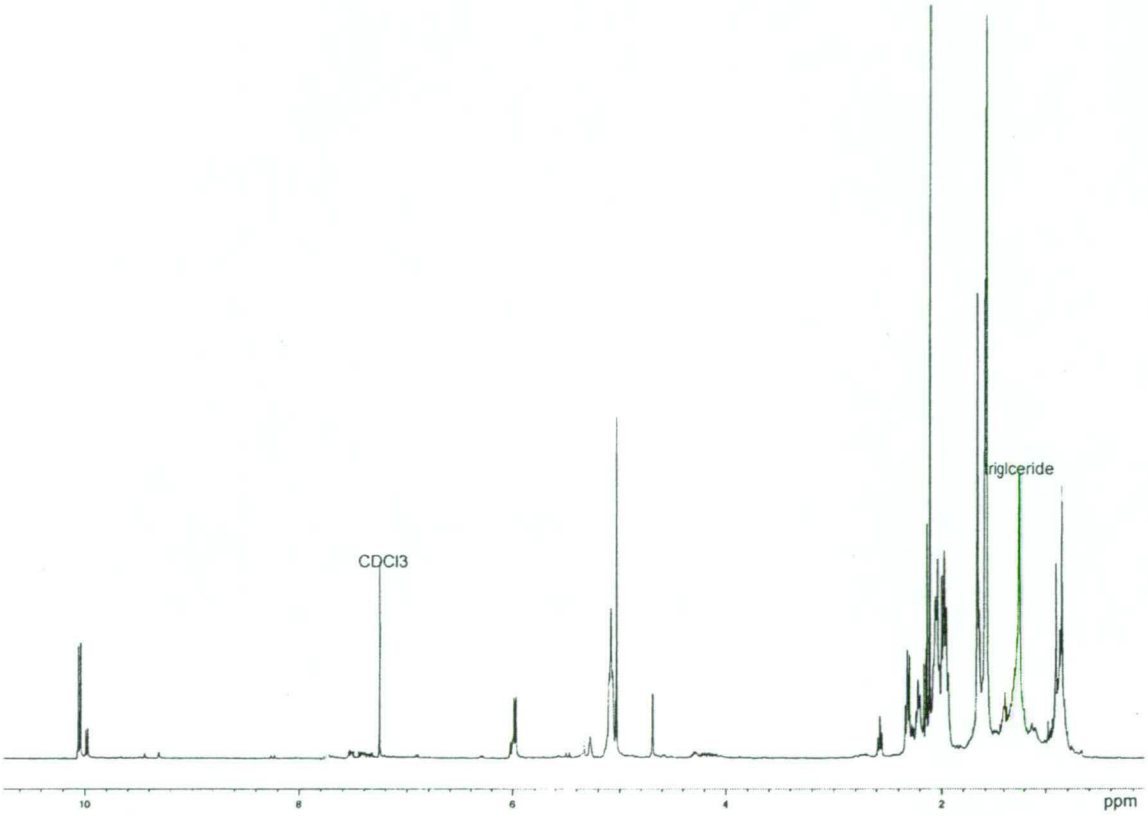


Figure 4.24 ^{1}H n.m.r. spectrum of (19) and (20) (400MHz, $CDCl_3$)

The l.c.-m.s. data for this fraction indicated the presence of a major **(19)** and a minor metabolite **(20)** with molecular weight 346 a.m.u. corresponding to a molecular formula of $C_{22}H_{32}O_4$. Of the four collections of *C. brownii* (unbranched) undertaken for this thesis, the major metabolite **(19)** was obtained in proportions from 95% to 50%. The ^{1}H n.m.r. spectrum of **(19)** is shown in Figure 4.29. Due to the small quantities of these metabolites present in the extract, other compounds of the same polarity such as triglycerides now became a problem. Although present in small amounts these

compounds gave rise to signals at 1.25 ppm in Figures 4.28 and 4.29. These triglycerides possessed low solubility in acetonitrile and hence had been primarily removed from the extract by filtration before h.p.l.c. For this fraction (less than 5 mg total from four collections) triglycerides were present in larger amounts as can be seen in the ^{1}H n.m.r. spectrum in Figure 4.29. Subsequent n.m.r. spectra (GHMQC, ^{13}C and DEPT) were run on the collection which gave rise to the spectrum in Figure 4.28, with the metabolites being present in the approximate ratio of 80:20 as this was the only fraction present in large enough amounts for useful carbon spectra to be obtained (2 mg). A COSY n.m.r. spectrum was run on the sample that contained the major metabolite **(19)** but peaks due to the triglyceride contamination can be seen in this spectrum (Appendix 2, Figure 2.14) however this spectrum did provide useful structural information.

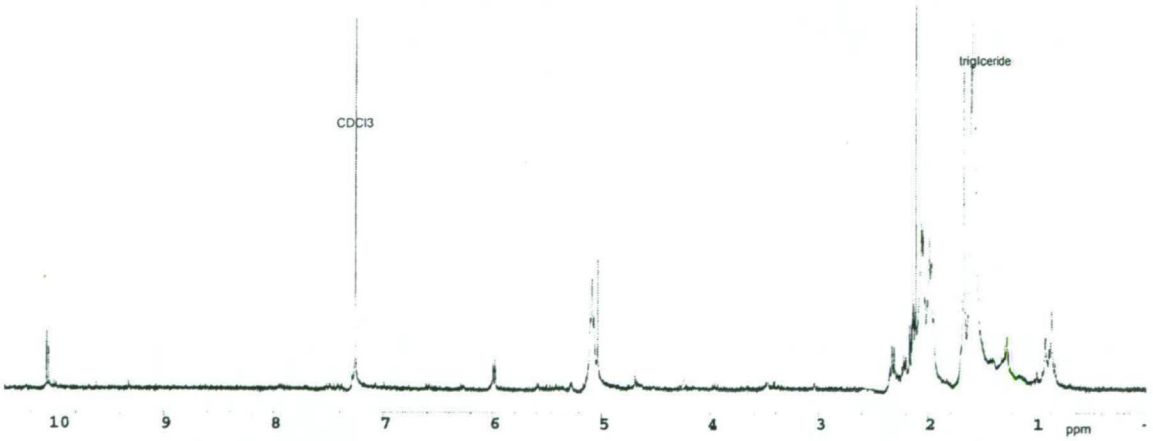


Figure 4.29 ^{1}H n.m.r. spectrum of (19) (400MHz, $CDCl_3$)

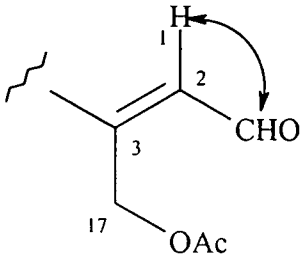
An inspection of the two ^{1}H n.m.r. spectra in Figures 4.28 and 4.29 suggests that the minor metabolite **(20)** possessed the monocyclic substructure as the signals previously attributed to geminal dimethyl groups at 0.90 ppm and 0.85 ppm were much diminished in Figure 4.29.

The ^{1}H n.m.r. spectrum of the major metabolite **(19)** contained a doublet at 10.05 ppm coupled to a doublet at 5.96 ppm, a singlet at 5.03 ppm which integrated for two protons and a sharp singlet at 2.11 ppm typical of an acetoxy methyl group. The minor metabolite **(20)** contained a similar set of signals at 9.98, 6.00, 4.69 and 2.13 ppm. The remainder of the ^{1}H n.m.r. spectrum shown in Figure 4.28 contained signals in the region below 2.6 ppm typical of the acyclic and cyclic moieties found in other fractions of *C. brownii* (unbranched) (Table 4.6).

The ^{13}C n.m.r. spectrum (Figure 4.30) included signals at 190.5 ppm typical of an aldehyde carbon, a signal at 170.1 ppm typical of an acyl carbon, a signal at 158.4 ppm typical of a carbon-carbon double bond adjacent to an aldehyde moiety and a signal at 61.5 ppm typical of a methylene carbon allylic to an acetoxy group.

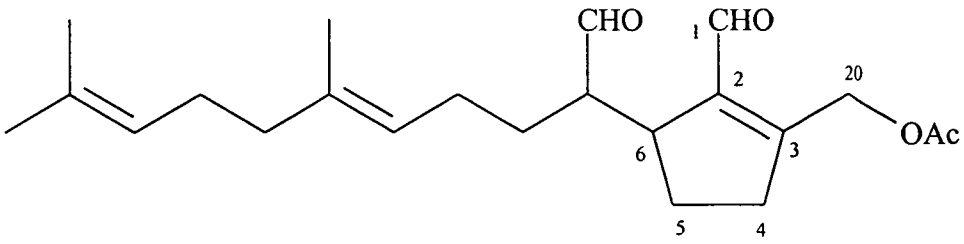
The ^{13}C n.m.r. spectrum also contained a number of signals in the region 122 – 137 ppm and signals below 49.1 ppm almost identical to the signals seen in the acyclic and cyclic metabolites previously found in *C. brownii* (unbranched) (Table 4.6). DEPT and GHMQC n.m.r. experiments confirmed the presence of the acyclic and monocyclic moieties present in previous fractions of *C. brownii* (unbranched) (Table 4.6). What was then required was to identify the C 1-C 5 moieties present in **(19)** and **(20)**.

The COSY n.m.r. data of **(19)** (Figure 4.31) showed a correlation between the aldehyde proton at 10.05 ppm and the vinyl proton at 5.96 ppm whilst the ^{13}C n.m.r. signals mentioned above were consistent with the C 1-C 3/17 substructure shown below:

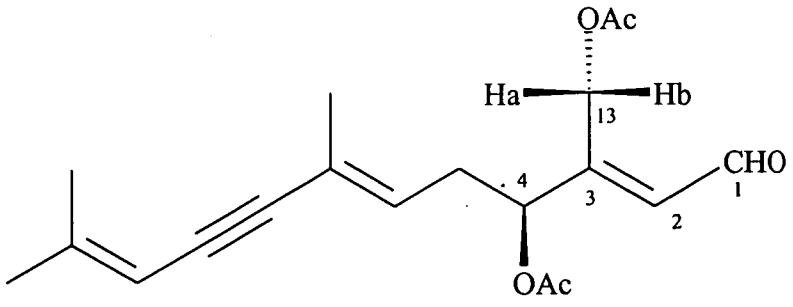


The H 17 protons at 5.03 ppm produced a sharp singlet consistent with the placement of the acetoxy group in this position whilst the *Z* steroechemistry of the C 2-C 3 double bond was consistent with the value of 10.05 ppm for the aldehyde proton (*E* = 9.3-9.4, *Z* = 10.0-10.1 ppm).[13]

The C 4 signal at 35.8 ppm was typical of a methylene carbon allylic to a double bond containing attached aldehyde and/or acetoxy groups (metabolites **(15)**, **(16)**). Two metabolites containing a similar moiety have been previously found in green algae. Petiodial **(38)** was isolated from the Mediterranean green algae *Udotea petiolata* collected from the Bay of Naples during the spring of 1982[22] and from *U. flabellum* collected from the Bahamas Islands in September 1981.[23] Taxifolial A **(39)** was isolated from *Caulerpa taxifolia* in 1992, collected from Cap Martin in the Mediterranean.[24]



(38)



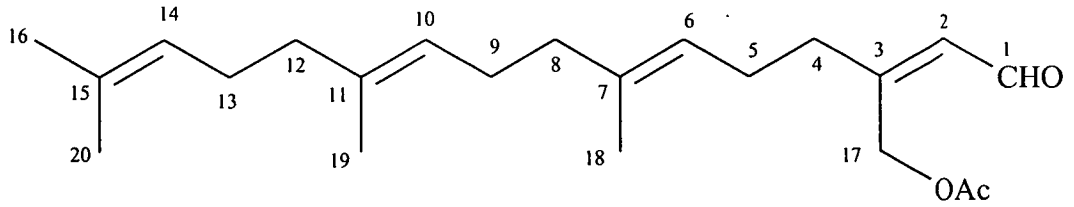
(39)

The ^{1}H and ^{13}C n.m.r. data for the C 1-3, C 13/20 moiety of these two metabolites and **(19)** and **(20)** are shown in Table 4.7 below and it can be seen that there is good agreement between the four metabolites.

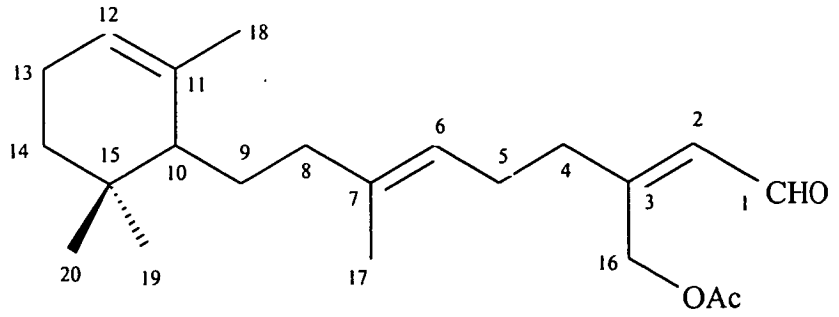
	(38)[a]		**(39)[b]**		**(19)**		**(20)**	
Carbon	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**
1	10.1	187.6	9.86	189.4	10.05	190.5	9.98	190.5
2	-----	140.6	6.03	128.9	5.96		6.00	
3	-----	158.4	-----	154.0	------	158.4	-----	158.4
$\underline{CH_2}OAc	5.06	59.6	4.75/4.59	59.1	5.05	61.5	4.69	61.5

Table 4.7 ^{1}H and ^{13}C n.m.r. data for metabolites (38), (39), (19) and (20); a=($CDCl_3$), b= (C_6D_6)

Thus the available evidence presented here is consistent with the structures below for **(19)** and **(20)**.



(19)



(20)

	(19)		(20)	
Carbon	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	190.5	10.05	190.5	9.98
2		5.96		6.00
3	158.4	-----	158.4	----
4	35.8	2.62	35.8	
5				
6	124.5	5.08	122.5	5.08
7	136.8	----	136.8	----
8	39.9	1.94-2.06	40.6	1.9
9	26.7	1.94-2.06	29.9	1.3
10	124.1	5.08	49.1	5.08
11	135.3	------	136.9	------
12	39.8	1.94-2.06	120.1	5.28
13	21.5	1.94-2.06	23.2	1.96
14	122.6	5.08	31.8	1.42/1.10
15	131.5	------	32.7	--------
16	25.9	1.6	61.5	4.69
17	61.5	5.03	16.3	1.58
18	16.2	1.65	23.6	1.59
19	17.8	1.66	27.6/7	0.85
20	16.1	1.67	27.6/7	0.90
$O\underline{C}OCH_3$	170.1	-----	170.1	-----
$OCO\underline{C}H_3$	20.7	2.11	20.7	2.13

Table 4.6 ^{1}H and ^{13}C n.m.r. data for metabolites (19) and (20) in $CDCl_3$

Isolating metabolites present in such small quantities proved to be a labour intensive exercise. However using techniques developed during the *C. flexilis* investigation it should now be possible to more completely remove the triglyceride compounds before analysis. A large collection of *C. brownii* (unbranched) dedicated to these metabolites should yield therefore a quantity of pure metabolites for further testing and analysis.

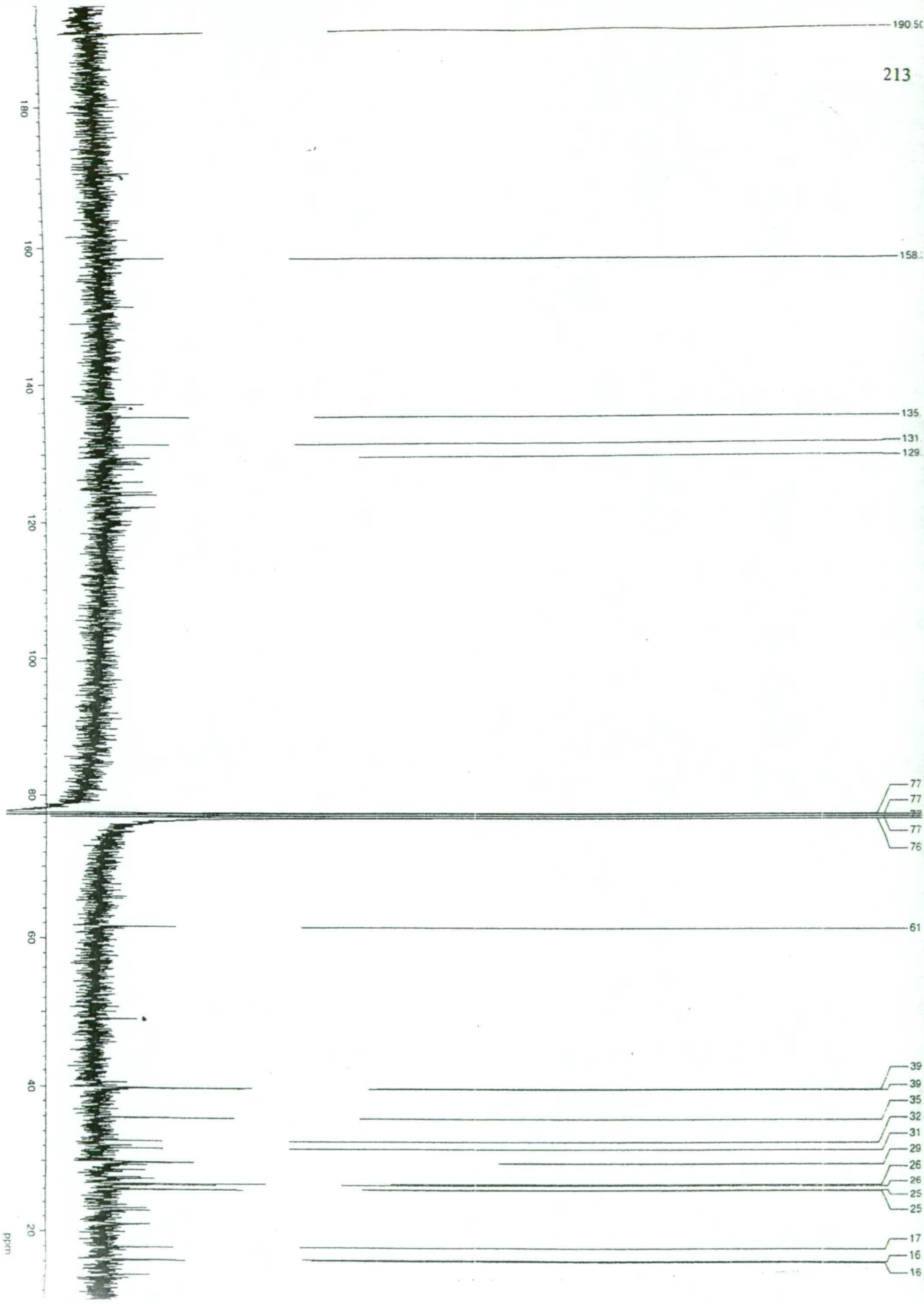


Figure 4.30 ^{13}C n.m.r. spectrum of (19) and (20) (100 MHz, $CDCl_3$)

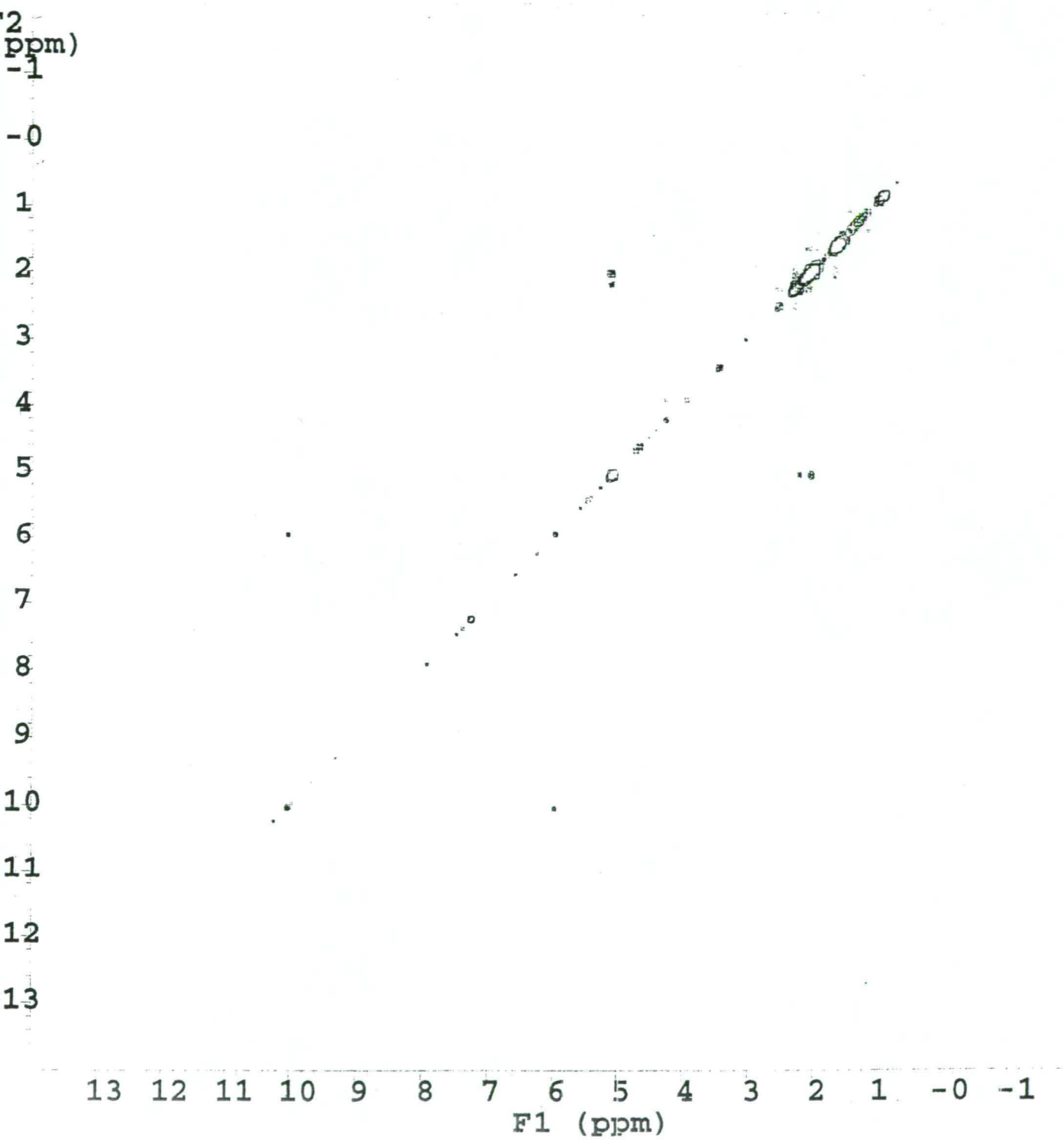


Figure 4.31 COSY n.m.r. spectrum of (19) (400 MHz, $CDCl_3$)

4.10 Structure elucidation of (21)

The most polar fraction obtained by p.t.l.c. was subjected to h.p.l.c. (C 18, 5 % EtOAc in MeOH) to yield **(3)** as a clear oil which gave a molecular ion of 406 by both g.c.-m.s. and l.c.-m.s. By high resolution c.i. mass spectrometry, **(21)** was found to have a molecular formula of $C_{24}H_{38}O_5$ (M+H 407.28119, M+H calc. 407.27968)

The e.i. mass spectrum of **(21)** was almost identical to that of **(6)** indicating that a metabolite of similar structure was present (Figure 4.32).

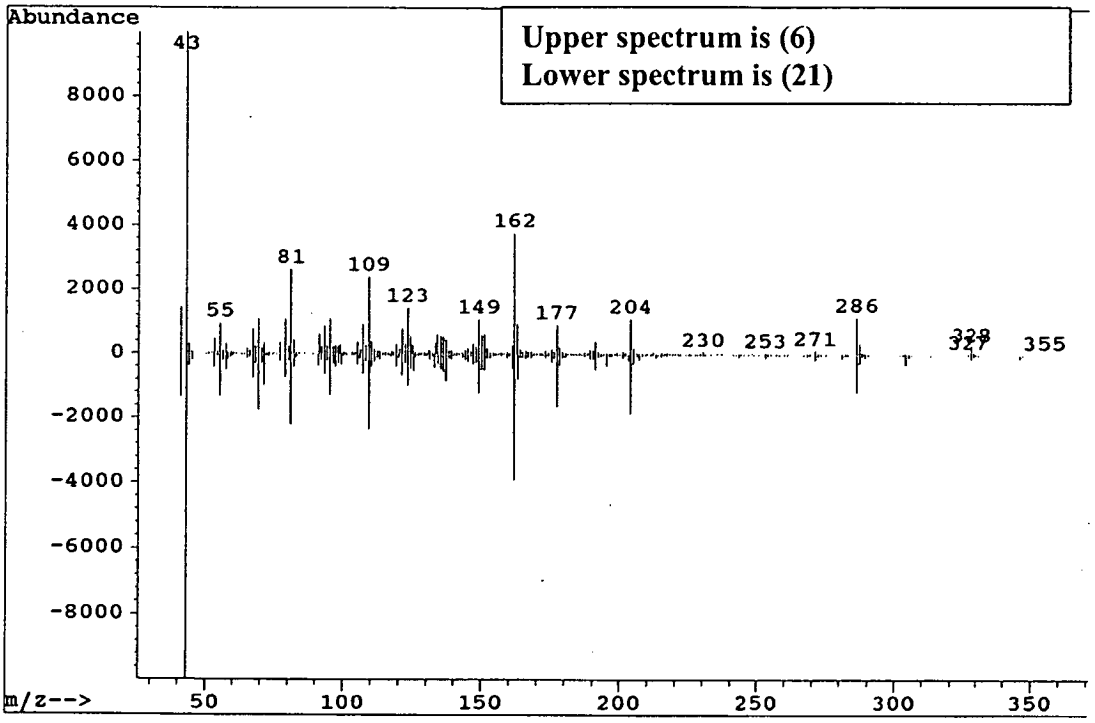


Figure 4.32 E.i. mass spectra of metabolites (6) and (21)

The ^1H n.m.r. spectrum of **(21)** in $CDCl_3$ contained signals characteristic of a 1,4-acetoxybutadiene moiety; two downfield protons at 7.46 and 7.17 ppm; the former a doublet coupled to a doublet at 5.94 ppm. The ^1H n.m.r. spectrum also contained six methyl signals at 2.17, 2.15, 1.62, 1.15, 0.94 and 0.82 ppm and methylene signals in the saturated aliphatic region between 1.0 and 2.4 ppm.

The ^{13}C n.m.r. spectrum of **(21)** contained twenty four resonances; two signals typical of carbonyl carbons at 168.1 ppm and 167.8 ppm, six signals in the vinyl region between 113 and 138 ppm, two signals at 74.3 ppm and 57.0 ppm and fourteen saturated aliphatic signals below 44 ppm. A DEPT experiment indicated that **(21)** possessed six methyl, seven methylene and five methine groups. Thus six quaternary carbon atoms were present, including the atom responsible for the signal at 74.3 ppm. The DEPT analysis accounted for thirty seven protons leaving one proton attached to an atom other than carbon. The quaternary carbon at 74.3 ppm possessed a chemical shift typical of a carbon attached to a oxygen atom leading us to suspect **(21)** possessed a tertiary alcohol functionality.

It is common for the mass spectrum of an aliphatic alcohol to closely resemble the coresponding alkene; hence it was possible that **(21)** was structurally similar to **(6)**; however possessing a hydroxyl group and an additional hydrogen atom rather than a carbon-carbon double bond. A closer inspection of the g.c. profile for **(21)** also showed a trailing edge to the peak (Figure 4.33); a common feature for compounds possessing an alcohol functionality.[10]

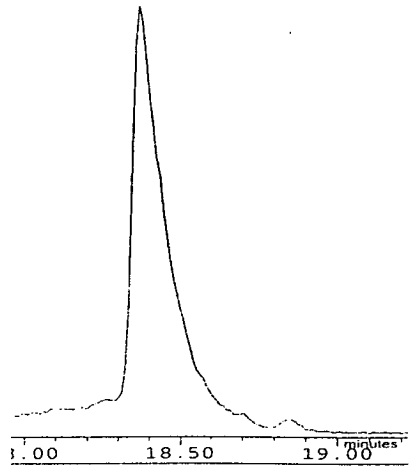
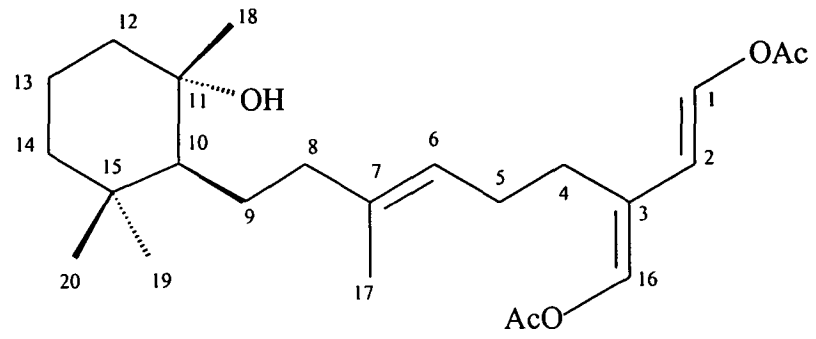
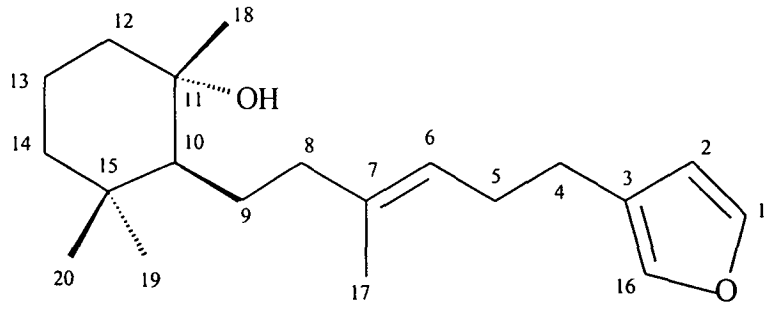


Figure 4.33 G.c. profile of (21)

The alcohol **(3)** was previously isolated from *C. brownii* by Paul and Fenical in 1985, who used ^{1}H n.m.r. decoupling studies of model compounds[11] and also comparative n.m.r. data from ambiol-A **(22)**[12] to aid in structure elucidation.



(3)



(22)

A comparison of the relevant n.m.r. data for **(3)**, **(21)** and ambiol-A **(22)** (Table 4.8) indicated that **(3)** and **(21)** were the same metabolite. However the availability of HETCOR, COSY and HMBC n.m.r. experiments in our investigation of this metabolite has allowed the revised assignment of the ^{1}H-^{13}C correlations including those correlations due to three sets of non-equivalent protons (Figure 4.34).

(22)[12] (3)[11] (21)

Carbon	^{13}C (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	142.8	136.1	7.43	135.8	7.44
2	111.3	113.5	5.93	113.5	5.93
3	125.2	121.3	----	121.4	----
4	28.8	27.1	2.32	25.4	2.32
5	25.3	25.7*	2.19	26.7	2.16
6	124.1	123.4	5.17	123.3	5.
7	137.0	137.5	----	137.3	----
8	43.9^{+}	43.8^{+}	1.73	43.6	1.74/1.31
9	25.3	25.3*	1.38	24.7	1.55/1.34
10	56.7	57.0	1.34	57.0	1.08
11	73.7	73.8	------	74.3	------
12	41.8^{+}	41.9^{+}	1.52	43.0	2.08
13	20.8	20.9	1.25	20.6	1.42
14	43.2^{+}	43.4^{+}	1.20	41.6	1.20/1.37
15	35.6	35.7	------	35.7	------
16	139.2	135.0	7.17	134.5	7.17
17	16.2	16.5	1.62	16.3	1.62
18	23.5	23.6	1.16	23.3	1.16
19	21.6	21.7	0.82	21.5	0.82
20	33.0	33.1	0.94	32.9	0.94
$O\underline{C}OCH_3$		167.5		167.8	
$O\underline{C}OCH_3$		167.0			
$OCO\underline{C}H_3$		2 x 20.2			

Table 4.8 ^{13}C and ^{1}H n.m.r. data for (22), (3) and (21). * $^{+}$= values interchangeable within column[11,12]

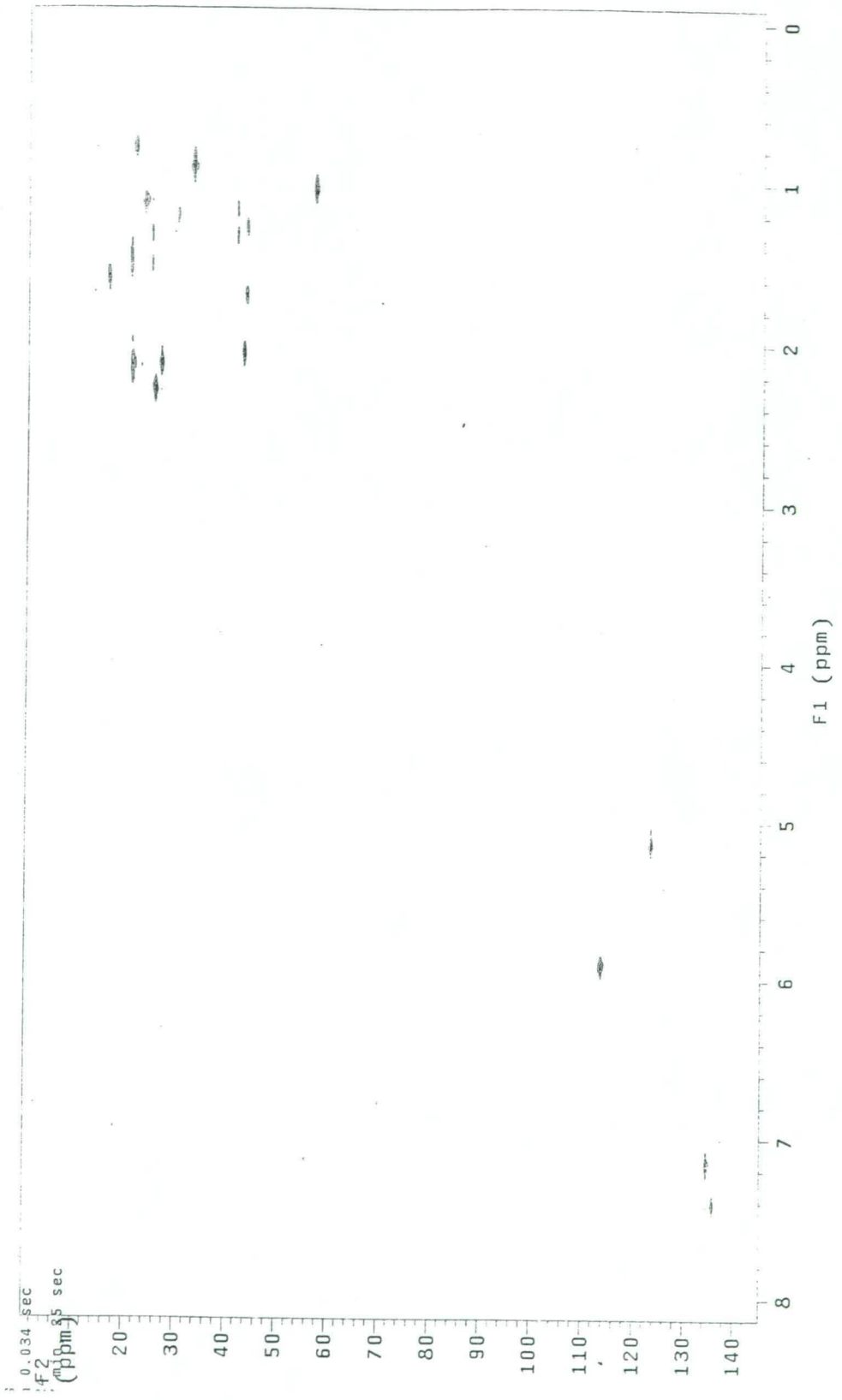
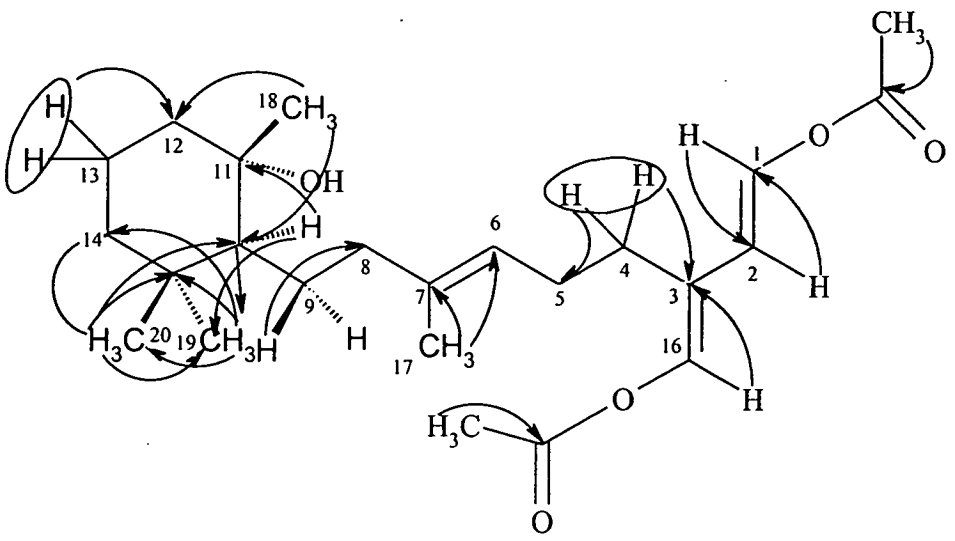
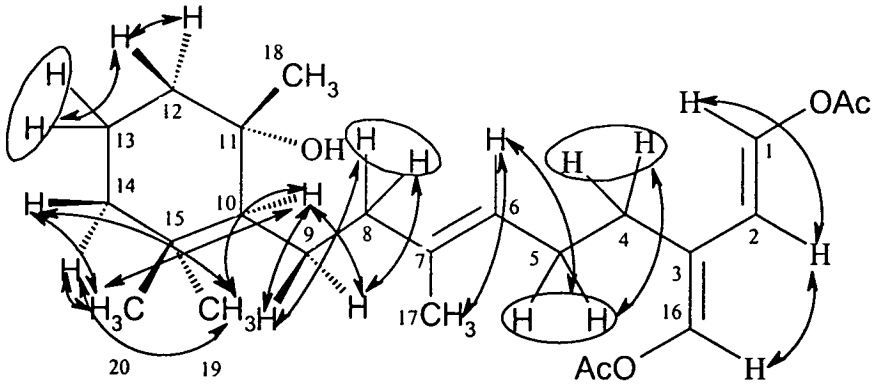


Figure 4.34 Partial HETCOR spectrum of (21) ($CDCl_3$)

The HMBC experiment (Figure 4.35) showed the following correlations:



Combining the HMBC correlations with the COSY correlations (Figure 4.36) shown below, further confirmed the structure of **(21)** and also enabled the correct positioning of the carbons at C 8, C 12 and C 14.



The presence of metabolites **(3)** and **(5)** and **(4)** in our investigation of *C. brownii* (unbranched) suggests that the *C. brownii* collected from Flinders Reef near Melbourne, Victoria by Paul and Fenical in 1985 (which was found to contain these three metabolites) was composed of **unbranched** specimens.

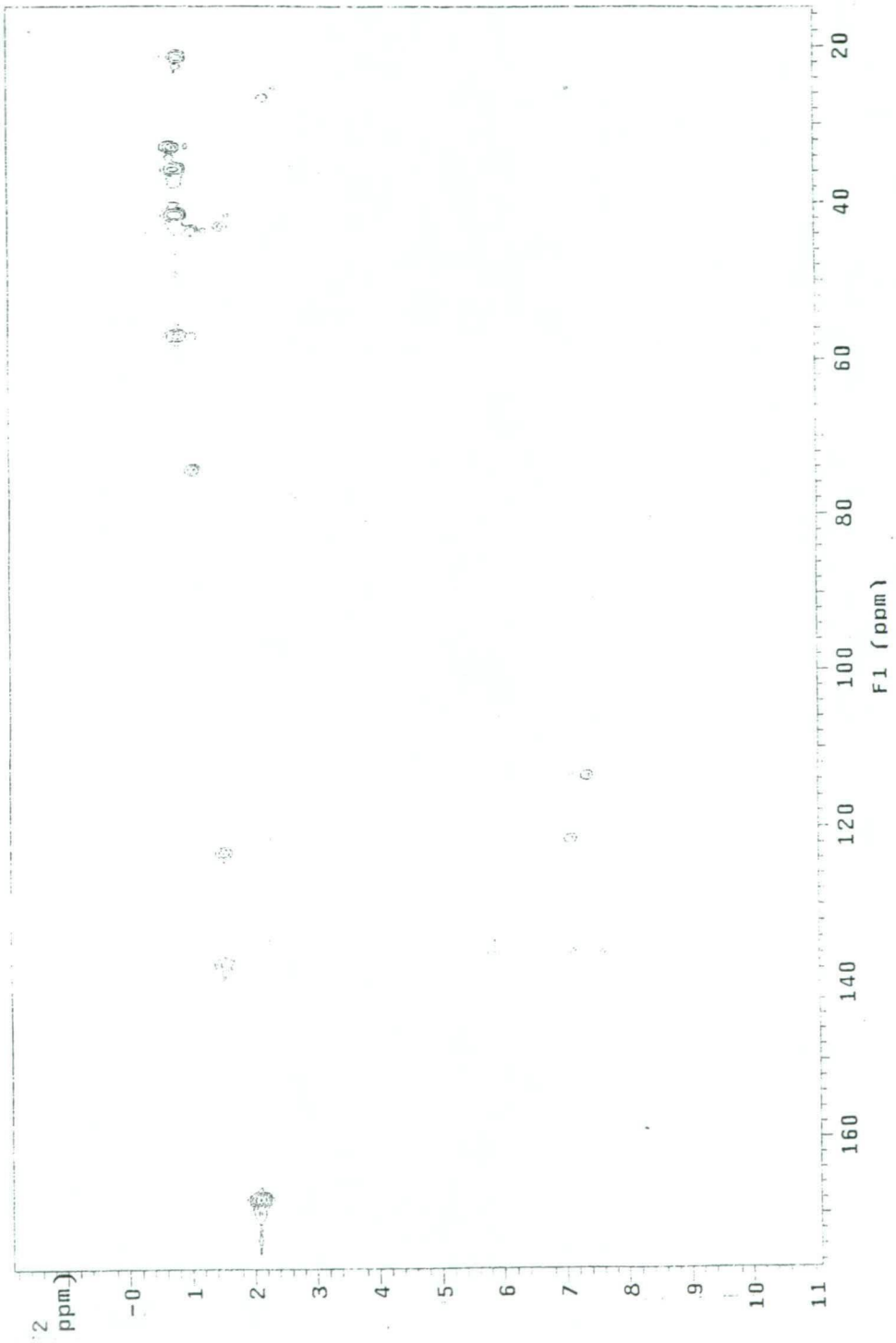


Figure 4.35 HMBC n.m.r. spectrum of (21) ($CDCl_3$)

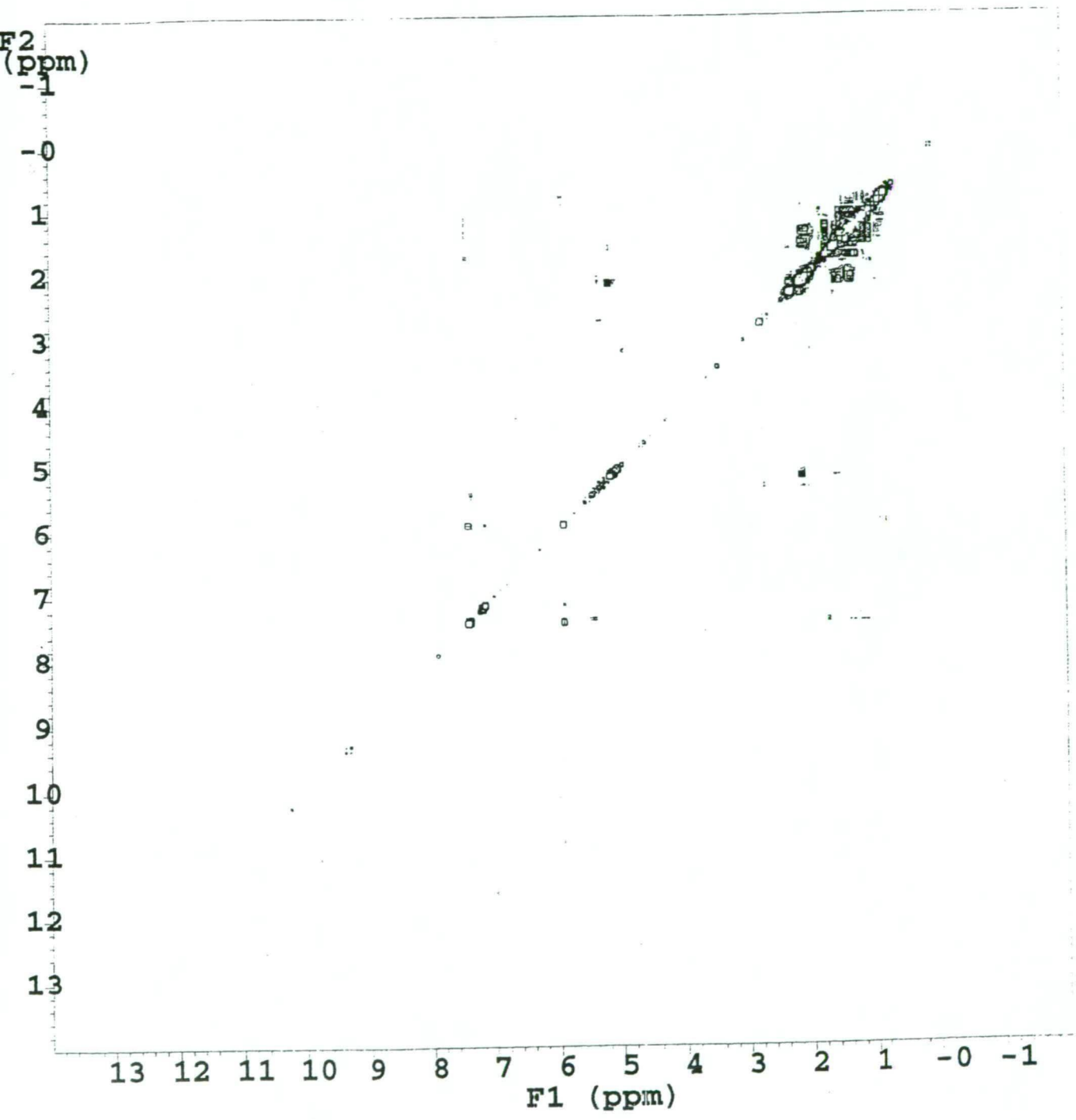


Figure 4.36 COSY n.m.r. spectrum of (21) (400 MHz, $CDCl_3$)

4.11 Caulerpol (1) and other metabolites of interest in *C. brownii* (unbranched)

At the completion of our investigation there remained two p.t.l.c. fractions of *C. brownii* (unbranched); these fractions were located between the fraction containing metabolites **(15)** - **(18)** and the fraction containing the alcohol **(3)**.

The first of these fractions (50 mg) contained a mixture of metabolites of molecular weight 360, 346 and 290 a.m.u. by l.c.-m.s. The ^{1}H n.m.r. spectrum showed a doublet at 4.12 ppm characteristic of C=CHC$\underline{H_2}$OH, signals typical of acyl methyl groups at 2.12 ppm and signals between 0.80 ppm and 2.30 ppm typical of both the acyclic and monocyclic metabolites found in other fractions of *C. brownii* (unbranched). Analysis of the mass spectral data for the metabolite of molecular weight 290 confirmed that it was caulerpol **(1)**. Subsequent h.p.l.c. analsysis (8 % EtOAc in pet. ether) achieved good peak separation however the small amounts involved resulted in collection of inadequate amounts for further analysis.

The second fraction (270 mg) contained a mixture of metabolites of molecular weight 360 and 332 a.m.u. by l.c.-m.s and triglycerides. The ^{1}H n.m.r. spectrum included signals typical of acetoxy methyl groups at 2.12 ppm and signals between 7.46 ppm and 5.88 ppm typical of acetoxybutadiene moieties.

Subsequent h.p.l.c. analysis (8 % EtOAc in pet. ether; 10 % H_2O in acetonitrile) achieved good peak separation however the small amounts involved after the removal of the triglycerides again resulted in the collection of inadequate amounts for analysis. A larger collection of *C. brownii* (unbranched) which is abundant at Spring Beach should lead to the successful isolation and identification of the metabolites in both these fractions.

4.12 Seasonal variation in *C. brownii* (unbranched)

Collections of *C. brownii* (unbranched) were made in the months of March, May, July and November from Spring Beach. Figure 4.37 below shows the g.c profiles for May 1999 and November 1999. It can be seen that the two profiles are almost identical which was also the case for the March and July collections, indicating that there is little seasonal variation in the metabolites that *C. brownii* produces.

Figure 4.37 G.c. profiles for May and November *C. brownii* (unbranched) collections

The ^{1}H n.m.r. spectra of the extracts of the different collections supported the conclusion that there was little seasonal variation in the production of metabolites in *C. brownii* (unbranched).

4.13 Temporal instability of metabolites of *C. brownii* (unbranched)

The metabolites present in *C. brownii* (unbranched) particularly those containing an acextoxybutadiene moiety proved to be unstable on storage. The figure below shows the two g.c. profiles of an August 97 collection; the lower profile is the g.c. profile of the extract immediately after freeze drying in August 97 whilst the upper profile is the g.c. profile of the remaining freeze dried seaweed which had been kept in the dark at -20^0C since August and extracted in November 97. Extracts were subjected to identical treatment before g.c. analysis.

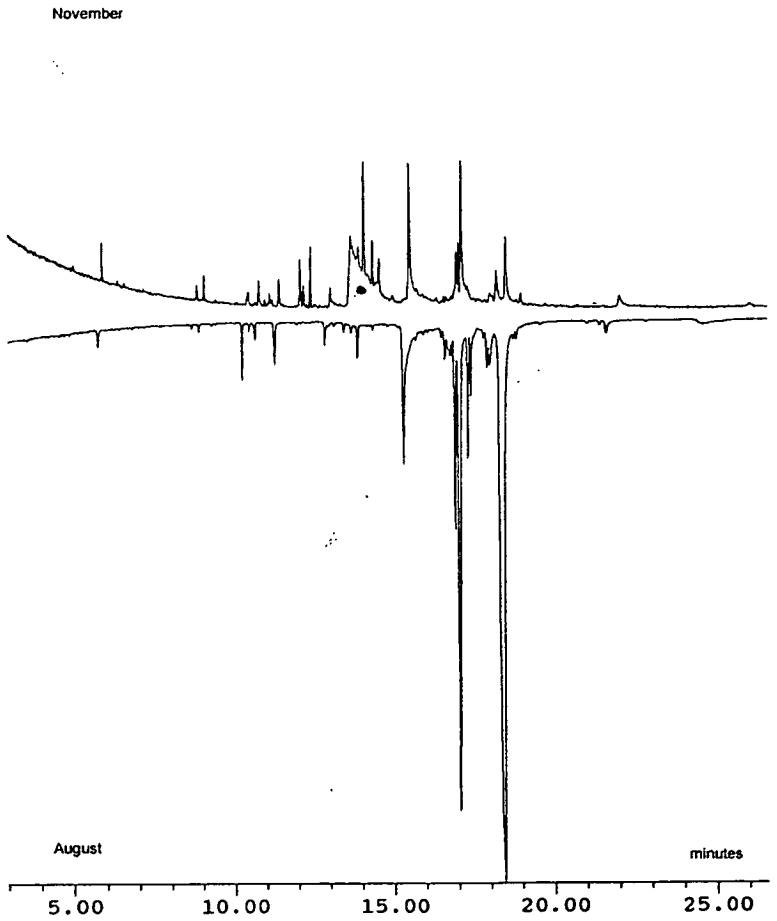


Figure 4.38 G.c. profiles of August and November extractions of *C. brownii* (unbranched) "•" = caulerpol time in minutes from *C. brownii* (branched)

The relevant ^{1}H n.m.r. spectra of the August and November extractions of the *C.browni* (unbranched) collections are shown below:

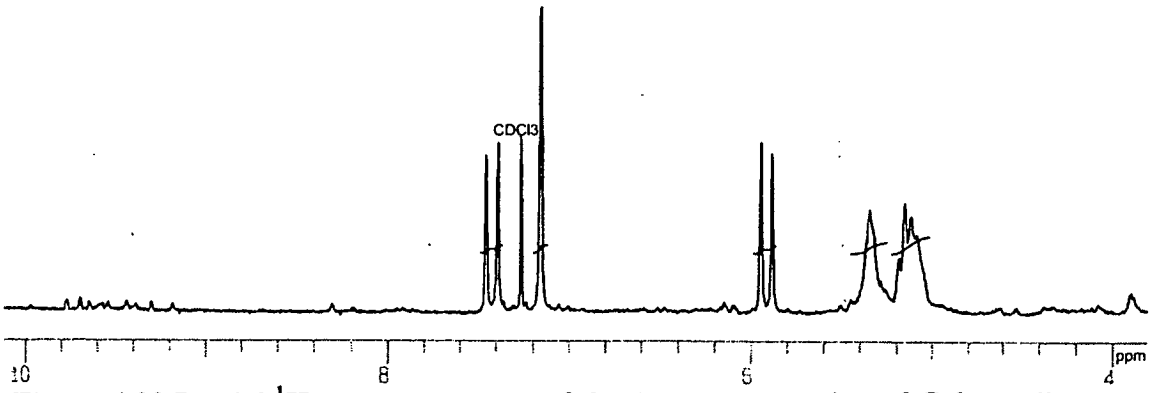


Figure 4.39 Partial ^{1}H n.m.r. spectrum of the August extraction of *C. brownii* (unbranched) (200MHz, $CDCl_3$)

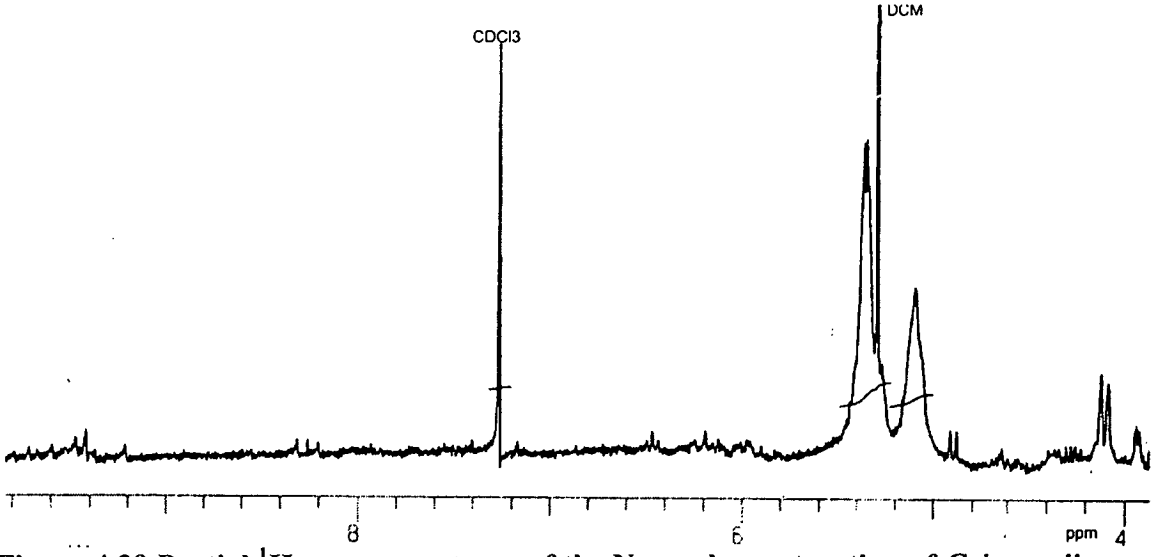


Figure 4.39 Partial ^{1}H n.m.r. spectrum of the November extraction of *C. brownii* (unbranched) (200MHz, $CDCl_3$)

It can be seen from an examination of these two ^{1}H n.m.r. spectra firstly that the signals due to acetoxybutadiene moieties have disappeared; (this was also found to be the case with similar metabolites in *C. trifaria*) and secondly signals typical of the $C\underline{H_2}OH$ moiety in caulerpol have appeared at 4.12 ppm.

The g.c. profile shown in Figure 4.38 suggested that caulerpol was present in the November extract in increased amounts; this was confirmed by m.s. evidence showing a m.s. data match for caulerpol for the peak at 13.8 minutes.

4.14 *C. brownii* (branched)

The branched specimens of *C. brownii* were extracted in an identical manner to the unbranched specimens, however the ^{1}H n.m.r. spectrum of the extract (Figure 4.39) and the g.c. profile (Figure 4.3) were quite different to that of the unbranched specimens.

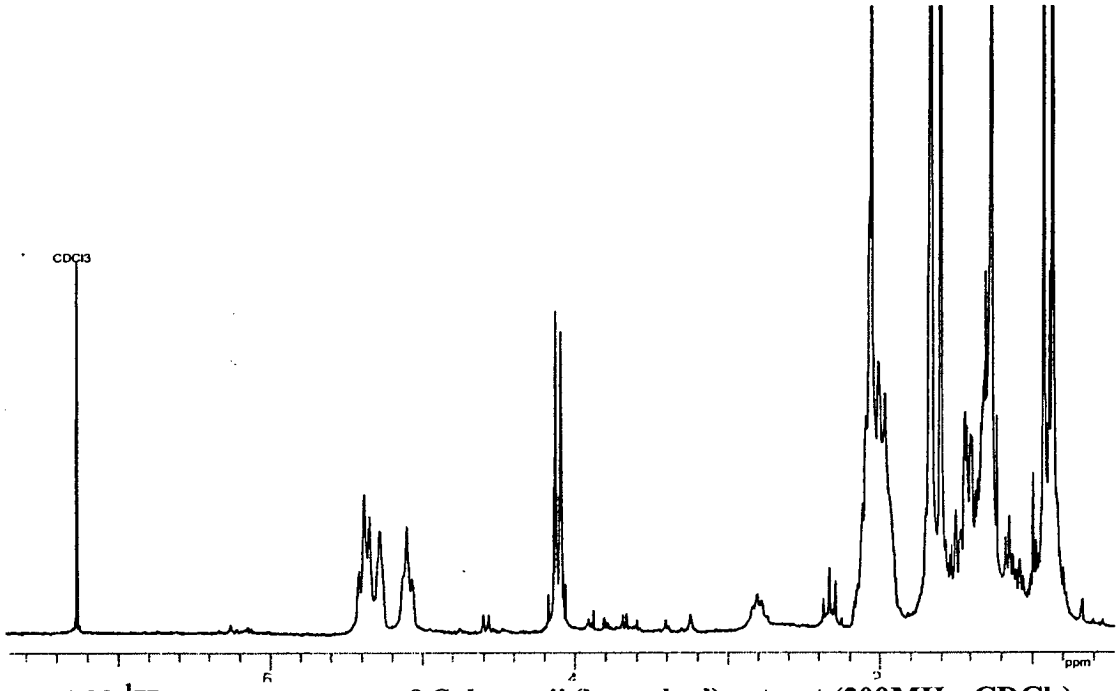


Figure 4.39 ^{1}H n.m.r. spectrum of *C. brownii* (branched) extract (200MHz, $CDCl_3$)

The t.l.c. of the extract of the branched specimens is shown below:

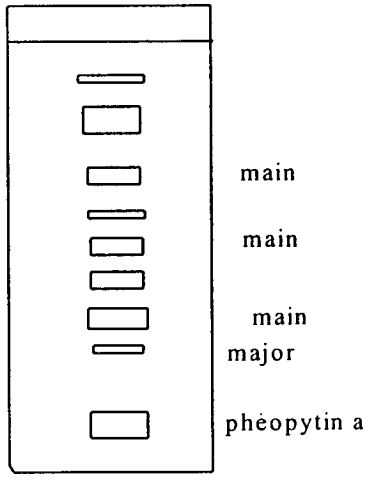
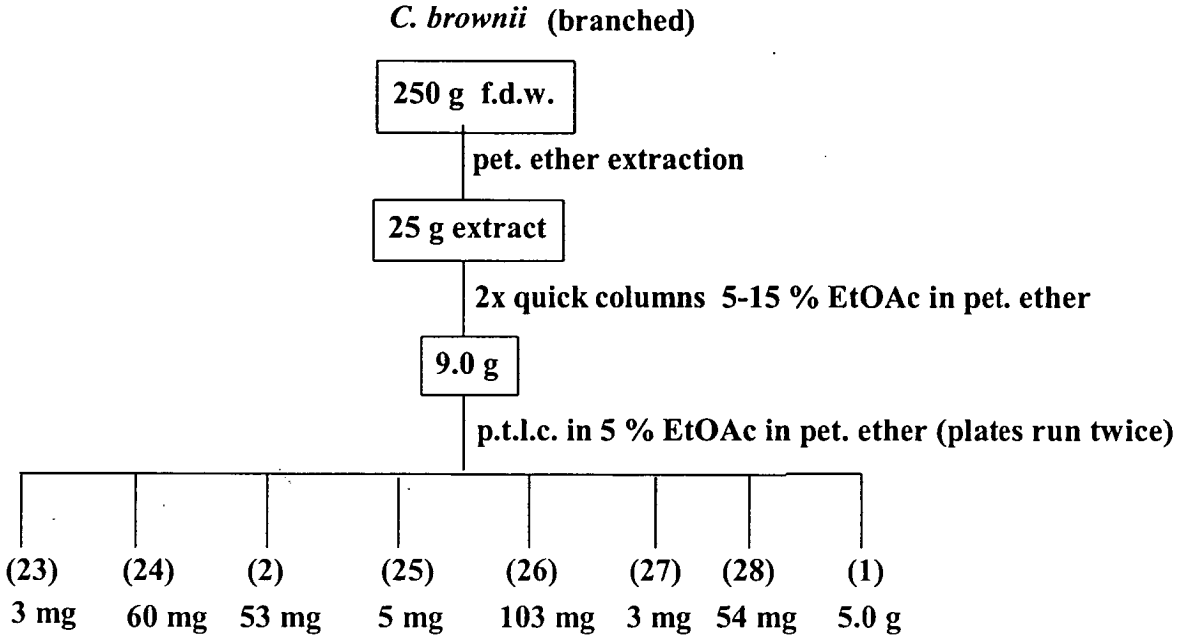


Figure 4.40 T.l.c. of branched specimens (5% EtOAc in pet. ether)

Subsequent p.t.l.c. (Scheme 4.2) resulted in the collection of eight fractions:



Scheme 4.2 Purification Scheme for *C. brownii* (branched)

4.15 Isolation of metabolites (1) and (2)

The major and most polar fraction obtained by p.t.l.c. consisted of one metabolite which gave a molecular ion of 290 by e.i. mass spectrometry corresponding to a molecular formula of C_{20} $H_{34}O$. The mass spectrum of this metabolite (Figure 4.41) possessed a fragmentation pattern suggestive of the monocyclic metabolites previously identified from *C. brownii* (unbranched) and *C. trifaria*.

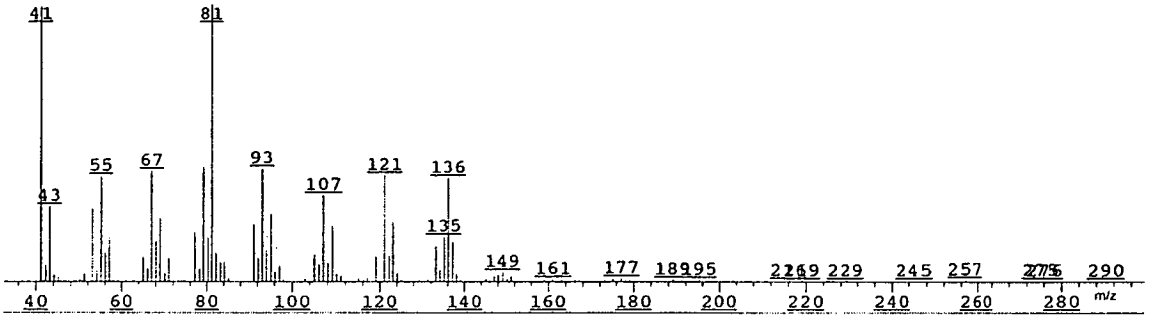
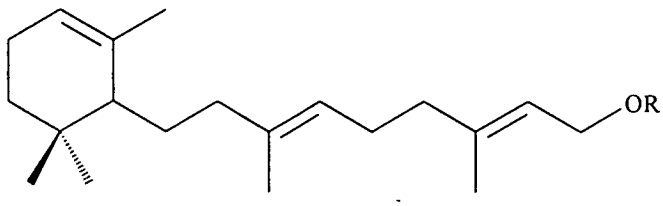


Figure 4.41 E.i. mass spectrum of major metabolite from *C. brownii* (branched)

The ^1H n.m.r. spectrum of this fraction (Appendix 2, Figure 2.17) included signals typical of a C=CHC$\underline{H_2}$OH moiety at 4.12 ppm, three signals in the region 5-5.4 ppm attributable to methine protons and signals at 1.61 ppm, 1.53 ppm, 0.85 ppm and 0.79 ppm typical of methyl group protons. An examination of the ^1H n.m.r. spectral data of caulerpol(**1**),[2] isolated by our research group in 1976 from *C. brownii* confirmed that the major metabolite isolated from the branched specimens in this investigation was also caulerpol (**1**).



(1) R=H (2) R=OAc

The g.c.-m.s. profile of the next fraction to be investigated indicated that one major metabolite was present with a molecular ion at 332 m/z. The mass spectrum (Figure 4.42) included a peak at 272 suggesting the presence of an acetoxy moiety and a fragmentation pattern typical of a monocyclic terpenoid.

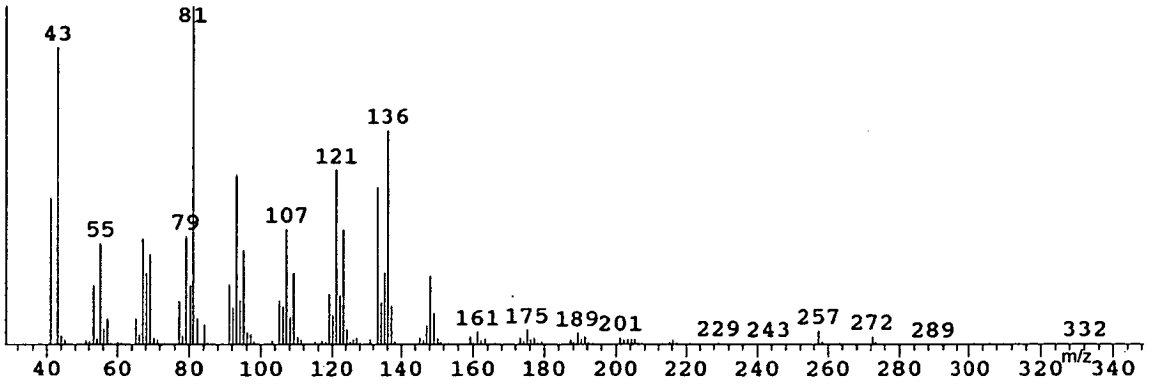


Figure 4.42 E.i. mass spectrum of (2)

The ^{1}H n.m.r. spectrum of this fraction was very similar to that of caulerpol **(1)** except the doublet at 4.12 ppm was now at 4.55 ppm, typical of a $C\underline{H_2}OAc$ moiety and a sharp peak at 2.03 ppm integrating to three protons was now present. A comparison of the ^{13}C and DEPT n.m.r. spectral data (Table 4.9) with corresponding data obtained in our 1976 investigation[2] indicated that the metabolite in this fraction was caulerpol acetate **(2)**. This was further confirmed by a COSY n.m.r. experiment.

	(2)		(23)	
Carbon	**^{13}C (ppm)**	**^{1}H(ppm)**	**^{13}C (ppm)**	**^{1}H(ppm)**
1	61.5 t	4.55 d *J* 7.2 Hz	61.3 t	4.55 d *J* 7.2 Hz
2	118.4 d	5.32 m	118.6 d	5.32 m
3	142.4 s	----	142.4 s	----
4	39.7 t	2.77 m	39.7 t	2.74 m
5	26.3 t	2.26 t *J* 7.6 Hz	26.3 t	2.26 t *J* 7.6 Hz
6	123.6 d	5.07 br s	123.6 d	5.05 br s
7	136.4 s	----	136.3 s	----
8	40.7 t	1.98 m	40.7 t	1.98
9	29.9 t	1.50/1.33 m	29.9 t	1.50/1.33 m
10	49.1 d	1.40 m	49.0 d	1.40 m
11	137.0 s	----	136.9 s	----
12	120.0 d	5.25 br s	120.0 d	5.25 br s
13	23.2 t	1.92-2.09 m	23.2 t	1.92-2.09 m
14	31.8 t	1.40/1.10 m	31.7 t	1.40/1.10 m
15	32.7 s	----	32.7 s	----
16	16.2 q	1.57 s	16.2 q	1.57 s
17	16.6 q	1.64 s	16.6 q	1.64 s
18	23.6 q	1.68 s	23.6 q	1.68 s
19	27.6 q	0.89 s	27.6 q	0.89 s
20	27.7 q	0.84 s	27.7 q	0.81 s
$O\underline{C}OCH_3$	171.3 s	----	174.0 s	----
$OCO\underline{C}H_3$	21.2 q	2.03 s		
Fatty acid			128-130.3**	5.35 m
residues			22-34.5*	1.25 m

Table 4.9 ^{13}C and ^{1}H n.m.r. data for (2) and (23) *=12 signals **=4 signals in this range

4.16 Esters of caulerpol (23)

The least polar fraction obtained by p.t.l.c. was investigated by l.c.-m.s. (Figure 4.43) resulting in the identification of six metabolites of molecular weight 522, 574, 550, 576, 552 and 554.

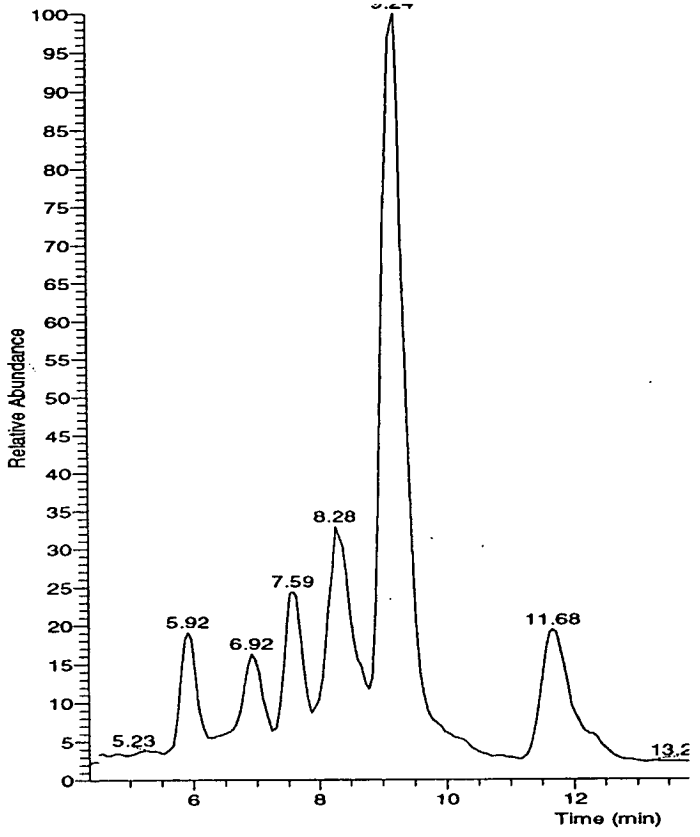


Figure 4.43 L.c. profile of least polar fraction of *C. brownii* (branched)

Table 4.10 shows the retention time (R.T.), molecular weight (MW) and mass spectral data of the metabolites in this fraction whilst the percentage composition is shown in Chart 4.1.

MW	R. T. (min)	Mass spectral data (APCI, C 18, 100% MeOH)
522	5.92	523(100), 399(7), 273(20)
574	6.92	575(100), 525(10),451(7), 305(7), 273(12)
550	7.59	551(100), 427(7), 273(18)
576	8.28	577(100), 553(7), 453(7), 305(5), 273(13)
552	9.24	553(100), 429(7), 273(18)
554	11.68	555(100), 529(12), 273(53)

Table 4.10 L.c.–m.s. data for the least polar fraction of *C. brownii* (branched)

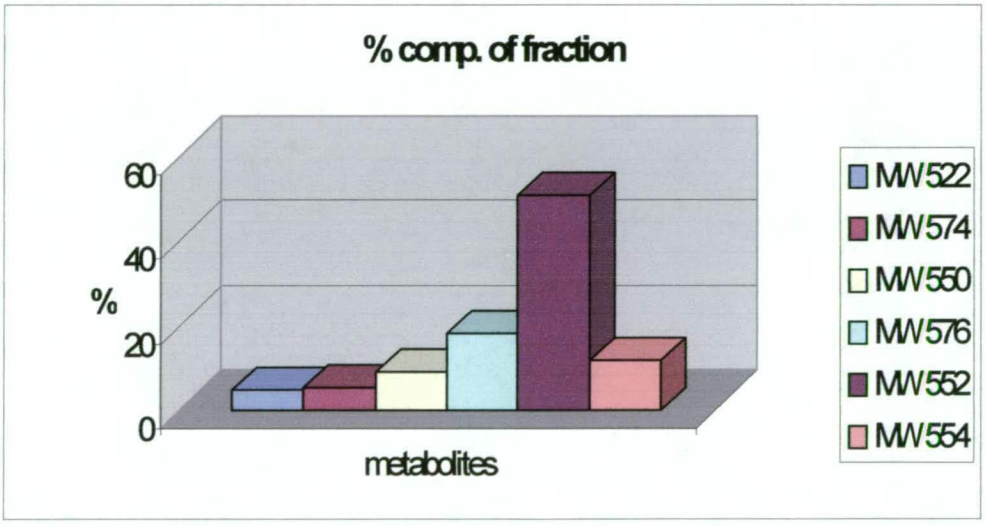


Chart 4.1 % comp. of metabolites in least polar fraction of *C. brownii* (branched)

The ^{1}H n.m.r. spectrum of this fraction (Figure 4.45) was very similar to that of caulerpol actetate **(2)** however the peak at 2.03 ppm (due to acetoxy protons) had disappeared whilst an intense signal at 1.26 ppm typical of saturated hydrocarbon long chains and a broadened signal at 5.35 ppm were now present.

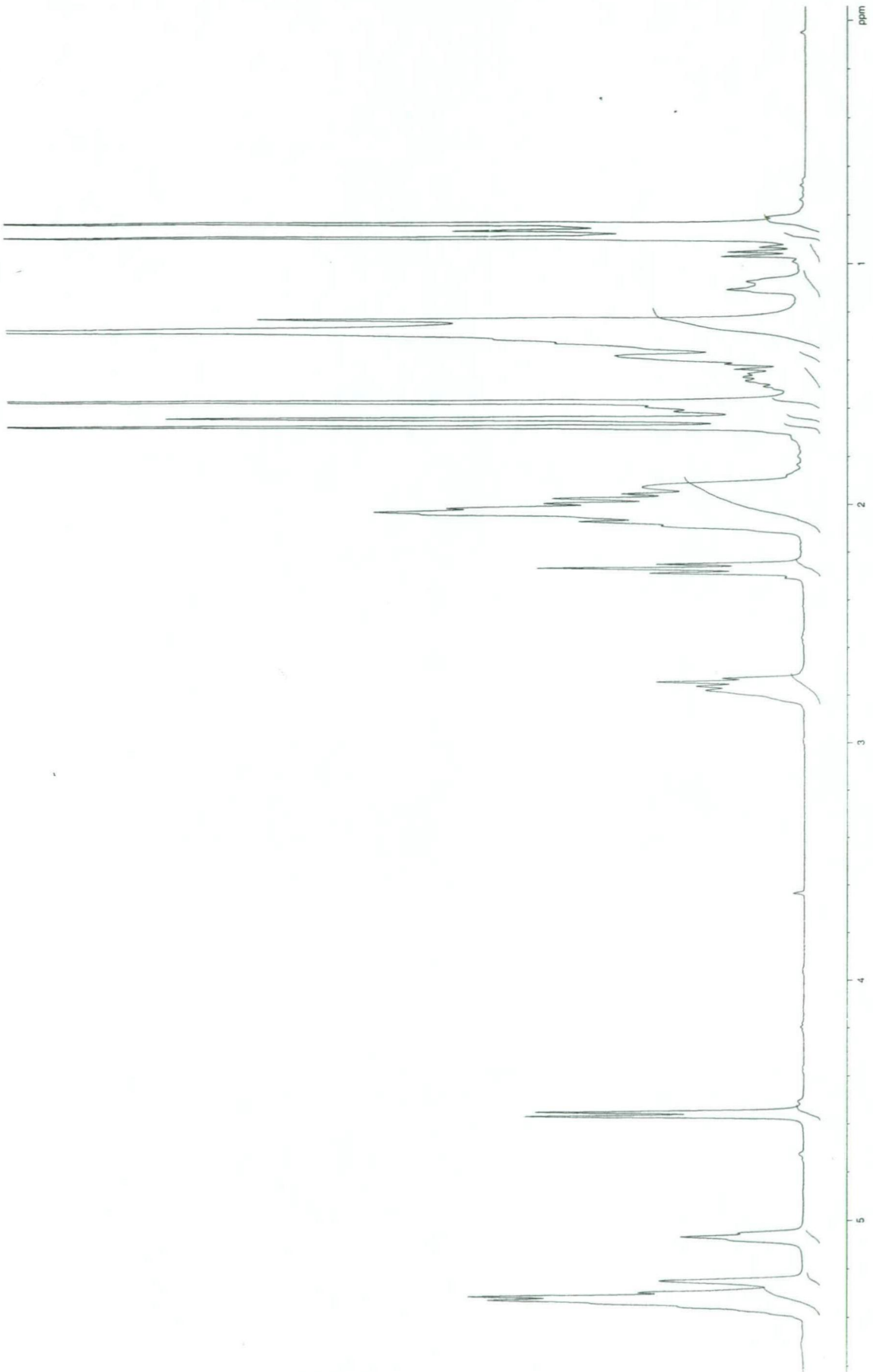


Figure 4.44 ^{1}H n.m.r. spectrum of least polar fraction (400 MHz, $CDCl_3$)

An examination of the ^{13}C n.m.r. spectrum (Figure 4.45) showed additional signals in the region 128-130 ppm and 15-30 ppm that were typical of fatty acid residues (Table 4.9) whilst the signal at 21.2 ppm (C 1) in **(2)** was missing. The remaining ^{13}C n.m.r. signals were almost identical to those in **(2)** with the exception of the quaternary signal at 174.0 ppm.

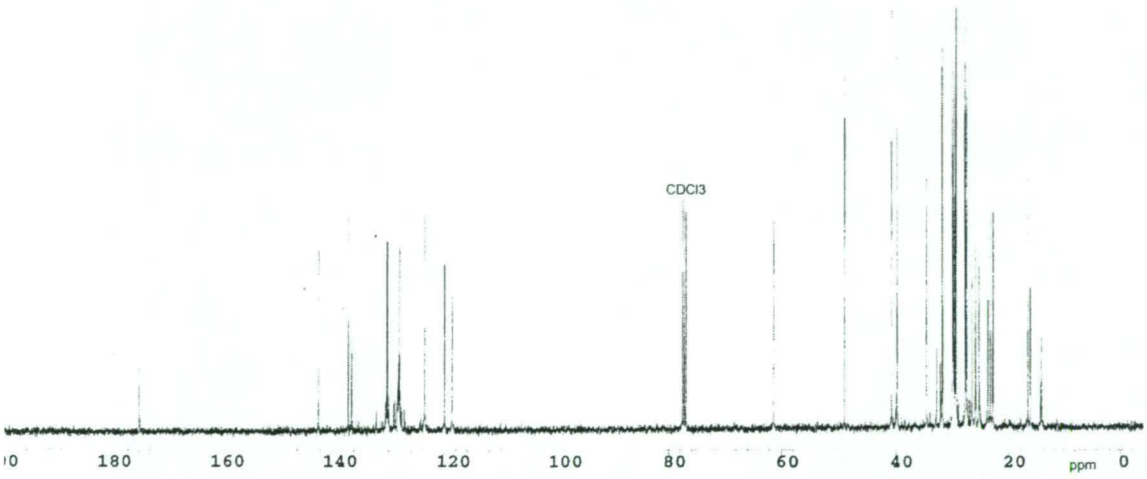
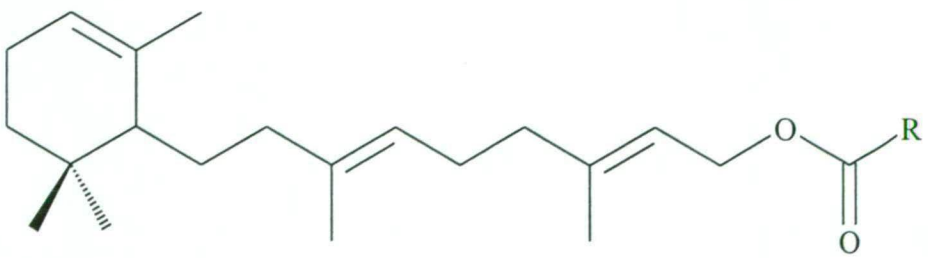
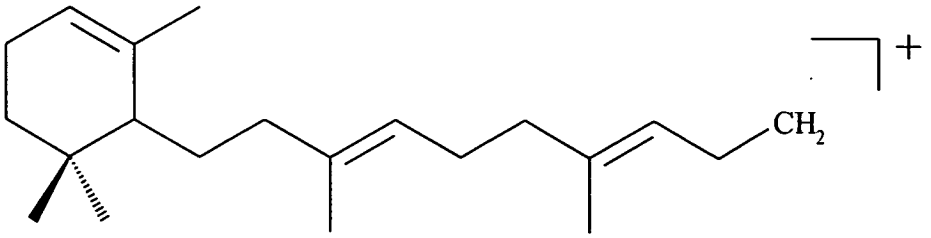


Figure 4.45 ^{13}C n.m.r. spectrum of least polar fraction (400 MHz, $CDCl_3$)

Thus the evidence presented so far supports the general structure below for the metabolites in this fraction, where the acetoxy group of **(2)** has been substituted by fatty acid residues:



A close examination of the fragmentation patterns obtained by l.c.-m.s. (Table 4.10) showed that all metabolites possessed a m/z ion at 273 (M+H- fatty acid) which corresponds to the fragment:



A comparison of the lc.-m.s. data of the six metabolites with the literature[14-16] on fatty acids confirmed that the following "R" groups were present (Table 4.11).

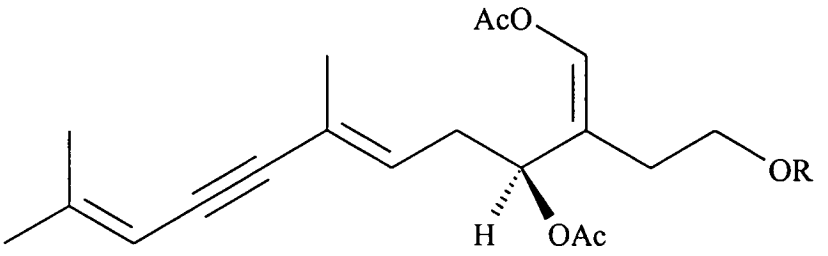
MW	R group	R group structure	Related fatty acid
576	$C_{19}H_{31}$-	$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_3$-	Arachidonic
574	$C_{19}H_{29}$-	$CH_3(CH_2)_2(CH{=}CHCH_2)_5CH_2$-	Eicosapentaenoic
554	$C_{17}H_{33}$-	$CH_3(CH_2)_7CH{=}CH(CH_2)_7$-	Oleic
552	$C_{17}H_{31}$-	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7$-	Linoleic
550	$C_{17}H_{29}$-	$CH_3(CH_2CH{=}CH)_3(CH_2)_7$-	Linolenic
522	$C_{15}H_{25}$-	$CH_3(CH_2)_4(CH{=}CHCH_2)_3CH_2$-	Hexadecatrienoic

Table 4.11 Fatty acid residues of metabolites in least polar fraction of *C. brownii* (branched)

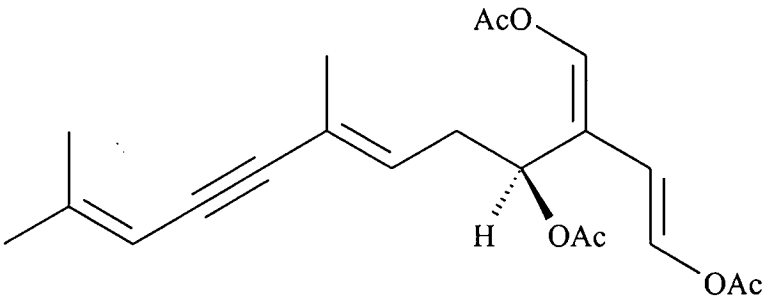
A recent investigation of the fatty acid content of *Caulerpa* species by capillary g.c. in 1995 by Kotimchenko[17] concluded that high concentrations of palmitic, α-linoleic, linoleic and hexadecatrienoic acids and low C_{16} C_{18} tetranoic acids were typical of this genus. It was also noted that *Caulerpa* species differ from other green algae by having a low C_{18} monosaturated acids content and approximately equal amounts of the ω9 and ω7 isomers.[17]

An investigation in 1982 into *C. prolifera*[18] resulted in the isolation of a didehydroderivative of caulerpenyne (**29**) with an acetoxy group substituted by fatty acid residues (**30**). This research group had previously isolated caulerpenyne (**29**) and

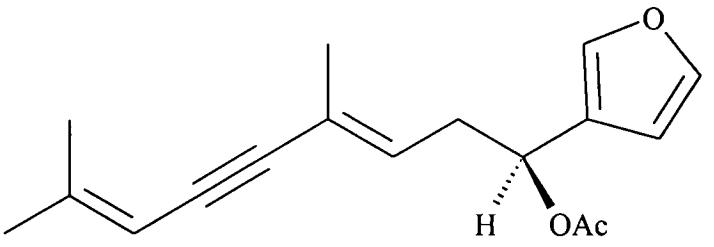
furocaulerpin **(31)** from this algae which is widely distributed in Mediterranean waters.[19,20]



(29) R= fatty acid residue



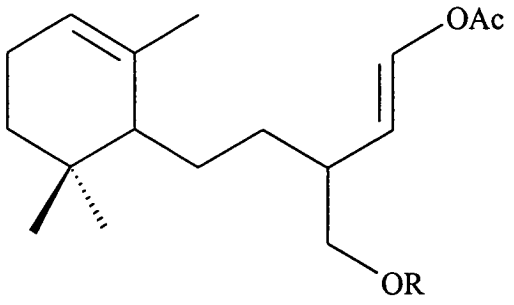
(30)



(31)

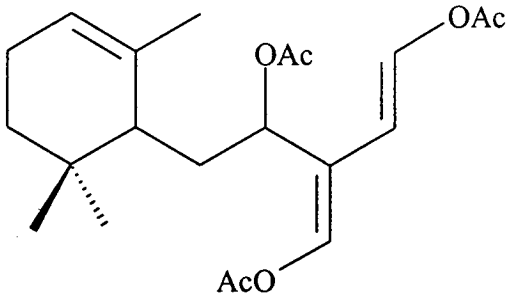
The caulerpenyne-like esters **(29)** were found to have the following composition of fatty acid residues: oleic 23%, linoleic 14%, palmitic 32%, palmitoleic 9% and myristic 22%.[18]

Two related sesquiterpenoid esters **(32)** (82%) and **(33)** (18%) have also been isolated from the tropical green alga *C. ashmeadii* by Paul and co-workers in 1987.[21]

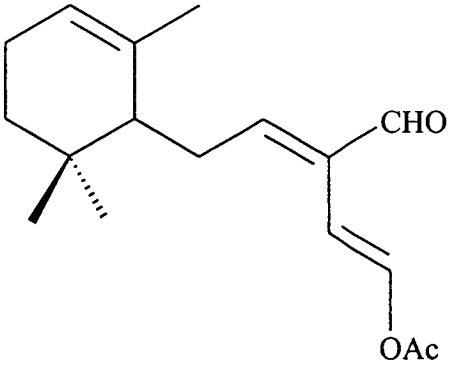


(32) R= $C_{16}H_{31}O$, **(33)** R= $C_{16}H_{29}O$

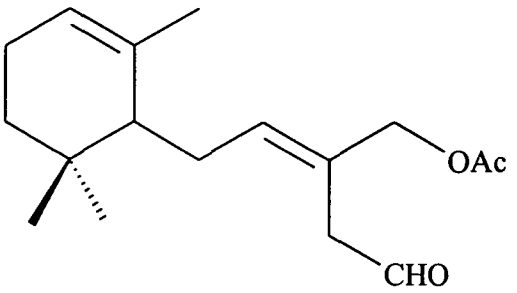
The novel metabolites **(34)**-**(37)** were also isolated from *C. ashmeadii* in this investigation. The metabolites **(32)** and **(33)** did not possess antimicrobial activity or toxicity towards damselfish whereas metabolites **(34)**-**(37)** possessed antimicrobial activity and toxicity towards the damselfish, with the aldehydes possessing the highest degree of biological actvity.[21]



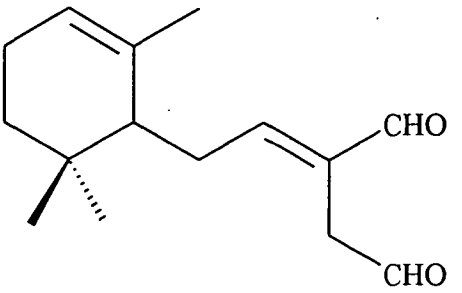
(34)



(35)



(36)



(37)

Thus the presence of the novel metabolites with fatty acid residues in *C. brownii* (branched) is consistent with the trend whereby the esters consist of another metabolite found in the alga which has had one of its acetoxy groups substituted by fatty acid residues.

4.17 Other compounds of interest in *C. brownii* (branched)

The remaining p.t.l.c. fractions of *C. brownii* (branched) **(24)**-**(27)** have not been investigated fully however the p.t.l.c. fractions **(24)** and **(27)** showed ^{1}H and ^{13}C n.m.r. spectra (Appendix 2) consistent with presence of other novel metabolite with fatty acid residues. Due to the curtailment of experimental work as explained in the Acknowledgements section of this thesis, these fractions remain unfinished. A re-investigation of the branched specimens of *C. brownii* (branched) should however lead to the identification of these metabolites.

4.18 Conclusions from *C. brownii* research

The conclusions that can be drawn from the foregoing work on *C. brownii* are firstly that the investigation into *C. brownii* by Paul and Fenical which yielded metabolites **(3)-(5)** was composed of **unbranched** specimens as these metabolites were amongst those isolated from our investigation into the unbranched specimens. Secondly the initial investigation twenty four years ago into *C. brownii*, which yielded caulerpol **(1)** and its acetate **(2)** was composed of **branched** specimens since our current investigation found these two metabolites to be the major metabolites present in the current investigation of the branched specimens.

Thirdly the presence of caulerpol **(1)** in trace amounts in the unbranched specimens indicates that there is some relationship between the two types of *C. brownii*. The fact that the amount of **(1)** increased noticeably in the stored extract of the unbranched specimens is also interesting and possibly suggests the conversion of other metabolites in the unbranched extract into **(1)**.

Fourthly, in addition to the known metabolites **(1)-(3)** *C. brownii* (unbranched) was found to be contain a number of novel metabolites with both acyclic and monocyclic moieties whilst *C. brownii* (branched) was found to contain the novel terpenoid esters **(23)**.

Finally *C. brownii* (unbranched) was found to contain a number of metabolites which were also found in *C. trifaria* suggesting common biosynthetic capabilities are present in both species of *Caulerpa*. This will be discussed in more detail in Chapter 7.

4.19 References

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